

DEPARTMENT OF OBSTETRICS AND GYNECOLOGY

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Date: March 3, 2000

Dockets Management Branch  
(HFA-305)  
Food and Drug Administration  
5630 Fisher Lane, Room 1061  
Rockville, MD 20852

**Re: Docket No. 99D-5199**  
**Draft Guidance Document for Absorbable Devices for Adhesion Reduction**

Dear Sir or Madam:

This letter is in response to the request for comments on the above referenced guidance document. The majority of the clinical questions were thoroughly discussed at the January 25 panel meeting and I concur with the experts that a reduction in adhesion formation in and of itself is of benefit. This could include reductions in incidence, extent or severity of adhesions. Having done adhesiolysis surgery in animal models, I would consider the complications that occur in animals due to the presence of cohesive dense adhesions would only be magnified in the clinical population. Therefore, I would request that the FDA consider a reduction of adhesion formation to be of clinical significance.

However, the main point of this memorandum is to address some of the difficulties that I see with the proposed animal studies. Of greatest interest to me is the means by which we can, in preclinical studies, address the magnitude of hazard associated with the use of adhesion prevention devices in the clearance of bacterial infections. This will be discussed in great detail in the accompanying document. There are also points to consider with the proposed reproductive studies to assess fertility and development and the assessment of the effect of adhesion prevention devices on the course of malignancy.

Thank you for your consideration of my concerns with the draft regulatory guidance in this area. I would be happy to provide any further information that you might require. Please feel free to contact me if you have any questions.

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**Infection Study:** It is the impression from the document that the suggested preclinical study would evaluate mortality and abscess formation after the initiation of bacterial peritonitis with a mixed bacterial flora. Historically within the field of immunotoxicology, assessment of the risk of alterations in immune function has been to evaluate a battery of immune parameters and to include, for comparison to evaluate the potential magnitude of the hazard, a host resistance assay. After a number of compounds were evaluated under this system of assessment of multiple parameters, then correlations were done to determine which immune function parameters are most predictive of alterations of host resistance and the effect of agents on these parameters were used to assess immunotoxic potential (Luster et al, 1993). The use of host resistance models to assess immunotoxicologic potential is rare in the regulatory arena. There are vast difficulties with the routine use of such models including the humane use of animals, the potential for interactions between the model pathogen and the chemical may give data not relevant to the clinical situation, the overwhelming nature of an infection leading to mortality in an animal model, etc.

With this historic background in mind, I would like to specifically address the draft protocol in question. It is unclear why the performance of studies of infection potentiation assessing mortality as the primary endpoint is recommended. As stated, within the field of immunotoxicology, host resistance studies are often assessed by the measurement of an important immune response parameter in the clearance of the agent of interest rather than mortality due to the infection. At a dose of infectious agent that results in significant mortality due to exposure, the immune system of the animal is often overwhelmed irrespective of the material undergoing evaluation (as evidenced by the mortality of the animal). At this point, it is difficult to determine the relevance to the human situation of, especially at a multiple increase in the clinical dose, the outcome of this study. The number of animals necessary at an LD50 dose that would be required to distinguish a clinically relevant event would be tremendous. I believe that any death that is directly related to the use of a product in a situation that is predictive of the clinical use of the product is relevant and would support the use of animals in the assessment of such a situation. However, measurement of mortality in a situation not relevant to the animal model used in preclinical assessment would not provide further comfort as to the safety of the material under evaluation.

On the other hand, intraperitoneal infections are often controlled by the host defense systems by abscess formation. Therefore, assessment of the effects of the materials on abscess formation may be of relevance to the clinical situation.

When I first came to this field of clinical research, I was asked, from my point of view as an immunotoxicologist, what would be the concern that I would have with the intraperitoneal use of an agent to reduce adhesion formation in the perioperative interval and what studies to conduct to determine if there was an increased risk with their use. I designed a set of studies that were subsequently published (Rodgers et al, 1988). This study assessed the function of peritoneal leukocytes including phagocytosis of opsonized yeast, respiratory burst activity and tumoricidal activity. Obviously, such an approach

would require validation as have occurred with the models to assess systemic immunotoxicity. However, ultimately this would provide data that would be more easily translatable to the human situation, and would not include some of the inherent risks of using a host resistance model.

However, if the need for a host resistance model continues, however, I would suggest that consideration be given to the use of a lower dose of bacterial inoculum with assessment of bacterial clearance and abscess formation as outcomes rather than mortality, which is so fraught with the potential for artifact.

Luster, MI, Portier, C, Pait, DG, Rosenthal, GJ, Germolec, DR, Corsini, E, Blaylock, BL, Pollock, P, Kouchi, Craig, W, et al. Risk assessment in immunotoxicology. II. Relationships between immune and host resistance tests. *Fund. Appl. Toxicology* 21:71-82.

Rodgers, KE, Ellefson, D, Girgis, W, Scott, L, diZerega, GS. 1988. Effects of tolmetin sodium dihydrate on normal and postsurgical peritoneal cell function. *Int. J. Immunopharmacology* 10:111-119.

**Reproductive Toxicology:** With the types of materials currently under development, many of the requested studies will require surgery on animal about to be mated or pregnant (or both if the sponsor would like to combine the studies into one). In fact, multiple surgeries may be required depending upon the pharmacokinetics of the abdominal clearance of the material. Surgery during pregnancy can be accomplished with rats. However, the ability to do this with a second species (rabbit) is not clear and the willingness of an animal undergo mating after recent surgery is not clear. The proper conduct of these studies, if possible (above comments reflect the most glaring problems), will be very expensive to the sponsor and would not provide additional evidence of safety of the product. As these agents are placed intraperitoneally and may act as a barrier to conception, infertility physicians generally do not recommend conception be attempted by their patients during the first cycle after surgery.

**Tumor metastasis:** While the above two protocols can be conducted and the relevance can be easily ascertained if there is a gross problem with the product, a review of available documents make it difficult to ascertain the proper conduct of a validated model to establish the effect of an agent on tumor metastasis. The only validated model of tumor metastasis used to evaluated host resistance in the field of immunotoxicology that I am aware of is a melanoma model that results in lung metastasis. This model is used to assess the effect of reduced systemic immune surveillance on the formation of nodules of metastatic tumor in the lung. However, how such a model can be made relevant to the intraperitoneal administration of a device that may reduce intraperitoneal immune surveillance or may allow tumor dissemination and metastasis after surgery is unclear.

Two models have been published in the literature that may provide the basis for a relevant, validated animal model for assessment of this safety parameter. These models are both in the rat and evaluate either ovarian cancer (Canis et al, 1998) or adenocarcinoma (Dorrance et al, 1999). The lack of a validated model (one that is not simply a laboratory method to evaluate a biological phenomena) would make compliance with this guidance point difficult.

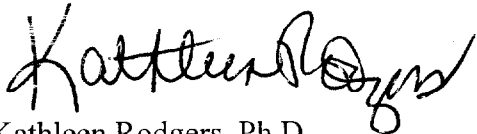
Canis, M., R. Botchorishvili, A. Wattiez, G Mage, JL Pouly and MA Bruhat. 1998. Tumor growth and dissemination after laparotomy and CO<sub>2</sub> pneumoperitoneum: A rat ovarian cancer model. Obstet. Gynecol. 92:104-108.

Dorrance, HR, K Oien, PJ O'Dwyer. 1999. Effects of laparoscopy on intraperitoneal tumor growth and distant metastases in an animal model. Surgery 126:35-40.

**Conclusions:** This section of the guidance, as currently written, is broad and vaguely defined. Before activating the guidance and consequently, requiring that studies of such a difficult and challenging nature be performed, I would strongly recommend that experts within the field meet as a committee to review this section of the guidance (as noted above I would volunteer to chair such a discussion with my colleagues). The committee would ensure that the recommended studies would be relevant and feasible to perform thereby eliminating any potential confusion amongst the community guided by this document.

Thank you for allowing me to have input on this document.

Sincerely yours,

A handwritten signature in black ink, appearing to read 'Kathleen Rodgers', with a stylized, cursive script.

Kathleen Rodgers, Ph.D.  
Associate Professor

## Risk Assessment in Immunotoxicology

### II. Relationships between Immune and Host Resistance Tests

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Risk Assessment in Immunotoxicology. II. Relationships between Immune and Host Resistance Tests. LUSTER, M. I., PORTIER, C., PAIT, D. G., ROSENTHAL, G. J., GERMOLEC, D. R., CORSINI, E., BLAYLOCK, B. L., POLLOCK, P., KOUCHI, Y., CRAIG, W., WHITE, K. L., MUNSON, A. E., AND COMMENT, C. E. (1993). *Fundam. Appl. Toxicol.* 21, 71-82.

We have reported on the design and content of a screening battery using a "tier" approach for detecting potential immunotoxic compounds in mice (Luster *et al.*, *Fundam. Appl. Toxicol.*, 10, 2-19, 1988). The data base generated from these studies, which consists of over 50 selected compounds, has been collected and analyzed in an attempt to improve future testing strategies and provide information to aid in developing future quantitative risk assessment for immunotoxicity. In a recent study it was shown that as few as two or three immune parameters were needed to predict immunotoxicants in mice (Luster *et al.*, *Fundam. Appl. Toxicol.*, 18, 200-210, 1992). In particular, enumeration of lymphocyte populations and quantitation of the T-dependent antibody response were particularly beneficial. Furthermore, commonly employed apical measures (e.g., leukocyte counts, lymphoid organ weights) were fairly insensitive. The present analyses focus on the use of this data base to develop statistical models that examine the qualitative and quantitative relationship(s) between the immune function and host resistance tests. The conclusion derived from these analyses are: (1) A good correlation exists between changes in the immune tests and altered host resistance in that there were no instances where host resistance was altered without affecting an immune test(s). However, in some instances immune changes occurred without corresponding changes in host resistance. (2) No single immune test could be identified which was fully predictive for altered host resistance, although most assays were relatively good indicators (i.e., >70%). Several others, such as proliferative re-

sponse to lipopolysaccharide and leukocyte counts, were found to be relatively poor indicators for host resistance changes. (3) The ability to resist infectious agent challenge is dependent upon the degrees of immunosuppression and the quantity of infectious agent administered. (4) Logistic and standard regression modeling using one extensive chemical data set from the immunosuppressive agent, cyclophosphamide, indicated that most immune function-host resistance relationships followed linear rather than linear-quadratic (threshold-like) models. For most of the relationships this could not be confirmed using a large chemical data set and, thus, a more mechanistically based approach for modeling will need to be developed. (5) Using this limited data set, methods were developed for modeling the precise quantitative relationships between changes in selected immune tests and host resistance tests. © 1993 Society of Toxicology.

Experimental data have been collected over the last 10 years by the National Toxicology Program (NTP) characterizing the potential of various environmental chemicals and therapeutics to suppress the immune system and alter host resistance. A description and rationale for the selection of a battery of immune tests for this screening process have previously been described (Luster *et al.*, 1988). Data from these studies, which now encompass over 50 compounds, have been analyzed in an attempt to improve future testing strategies and provide information to aid in the risk assessment process. Specifically, the following issues were addressed: (1) What is the likelihood for each of the individual tests and testing configurations to accurately identify immunotoxic compounds? (2) How do these immune endpoints relate to other endpoints such as carcinogenicity? And (3) what are the quantitative and qualitative relationships between the immune tests and host resistance assays? The first two questions were addressed in an earlier study (Luster *et al.*, 1992) and statistical analyses indicated that in most instances the performance of only two or three im-

<sup>1</sup> D. Gayla Pait was on a student appointment from the Department of Mathematics, North Carolina State University, Raleigh, NC 27695 and is presently at Glaxo, Inc., Research Triangle Park, NC 27709.

mune tests were sufficient to identify immunotoxic compounds in rodents. The tests which showed the highest association with immunotoxicity were the splenic antibody plaque forming cell (PFC) response (78% concordance) and cell surface marker analysis (83% concordance). The relationships between immunotoxicity and carcinogenicity were also addressed. These analyses suggested that many immunotoxic compounds were likely to be rodent carcinogens, however, for compounds that were not immunotoxic the carcinogenic status was unclear. There was no apparent relationship observed between immunotoxicity and mutagenicity.

The present studies focus on the use of this data base to develop statistical models that examine the relationships between immune function and host resistance tests. While limitations exist in using such a data base, at present human and animal studies have offered only limited insight into identifying the most appropriate immune endpoints for predicting clinical disease or establishing the quantitative relationships between immune function changes and impairment of host defenses, although qualitative relationships have been shown. In addition to analyzing the 51 chemical data base, results of which were summarized in a preceding report (Luster *et al.*, 1992), a study was designed and the resulting data were collected to further examine these quantitative relationships. In this latter study, groups of mice were treated with a broad-spectrum immunosuppressant, cyclophosphamide, employing a larger number of dose levels than are generally used in immunotoxicity testing. The study design provided an opportunity to determine the relationship between immune function assays and certain host resistance parameters, particularly at the low end of the dose-response curve.

## MATERIALS AND METHODS

**Chemical data base.** Details of the NTP data base used for these analyses, including the results, have been previously described (Luster *et al.*, 1992). Briefly, this data set consisted of 51 chemicals or drugs examined by the NTP or NTP-sponsored laboratories since 1980. In addition, results from several of the compounds studied were provided by Dr. Jack Dean while at the Chemical Industry Institute of Toxicology (Research Triangle Park, NC). At present, there is no accepted definition of what constitutes an immunotoxic agent in rodents, although most agree that immunotoxic compounds would best be classified as those that produce an immune alteration potentially leading to an undesirable effect (e.g., infectious disease). For the purposes of these studies, a positive immunotoxic response was defined on the basis that the test material either produced a significant dose-response effect ( $p < 0.05$ ) or significantly ( $p < 0.05$ ) altered two or more test parameters at the highest dose of chemical tested. We perceive this as a "liberal" criteria for immunotoxicity, but appropriate for its intended use in the analyses. Of the 51 compounds included in the data base, 34 were classified as immunotoxic by these criteria (Luster *et al.*, 1992). For host resistance data, statistical significance was assessed by Fisher's exact test and/or the proportional hazards general linear model. Significance ( $p < 0.05$ ) at the high dose level was necessary to qualify as "positive." The results of the host resistance tests from the 51 chemical data base are summarized in Appendix A.

All studies were conducted using either B6C3F1 or C57BL/6 female mice that were between 8–10 weeks of age at the start of the study and most often employed three treatment levels plus controls. Group sizes routinely averaged 7 mice per treatment group for immune tests and 12 mice per treatment group for host resistance tests except the cyclophosphamide data set to be discussed later. The mice were maintained under pathogen-free conditions and routinely screened for the presence of hepatitis and Sendai virus.

**Cyclophosphamide studies.** In addition to the NTP data base, two additional analyses were conducted on data sets obtained using a large number of mice exposed to cyclophosphamide (Sigma Chemical Corp.) at multiple dose levels. In one study, groups of 20 mice were administered a single ip injection of 0, 50, or 200 mg/kg of cyclophosphamide in saline. Forty-eight hours later, each mouse received  $1 \times 10^4$ ,  $2 \times 10^4$ ,  $4 \times 10^4$  or  $8 \times 10^4$  PYB6 tumor cells by subcutaneous injection. Tumor appearance was monitored over a 60-day period and analyzed as described in the text. Data from a second study were used to develop several of the statistical models used in the studies. For this study, groups of B6C3F1 mice were administered a single intraperitoneal injection of cyclophosphamide at dose levels of 25, 50, 75, 100, 125, 150, 175, or 200 mg/kg and the immune or host resistance assessments were initiated within 48 hr. Only selected immune and host resistance tests were conducted in this study and included the following assays: plaque-forming cells (PFCs), cytotoxic T lymphocytes (CTL), mixed leukocyte response (MLR), mitogenesis, surface markers, spleen cellularity, thymus:body weight ratios, *Listeria monocytogenes* resistance, *Streptococcus pneumoniae* resistance and PYB6 tumor development. Details of the methods have been described (Luster *et al.*, 1988; Dean *et al.*, 1989; Munson and White, 1990; Thomas *et al.*, 1990). For some tests, the data were pooled from replicate experiments when allowed by analysis of variance.

**Screening battery.** The immune and host resistance tests analyzed from the 51 chemical data set are listed in Table 1, and detailed methodologies are described elsewhere (Luster *et al.*, 1988; Dean *et al.*, 1989; Munson and White, 1990; Thomas *et al.*, 1990). Briefly, this panel consisted of two tiers. Tier I included examination of lymphoid organs (e.g., histology, weights), hematological studies and tests for general immune function such as a modification of the antibody PFC assay originally described by Jerne *et al.*, (1963) and the quantitation of natural killer (NK) cell activity. Tier II provided a more in-depth study and included, among other tests, evaluation of effector T lymphocyte function (i.e., delayed hypersensitivity or cytotoxic T lymphocyte responses) and quantitation of splenic B lymphocytes, T lymphocytes, and T cell subsets. In addition, Tier II included host resistance models. In these assays, groups of experimental animals were challenged with either an infectious agent or transplantable tumor cells in an amount sufficient to produce a clinical or observable change in a small number of the control animals [e.g., yielding 20% morbidity rate (MD<sub>20</sub>) or tumor frequency (TD<sub>20</sub>) in the control group]. The host resistance models used in Tier II, along with the endpoints measured are listed in Table 1. It should be noted that as a laboratory test for immunocompetence, these assays historically are assumed to lack sensitivity, most notably because they provide categorical data (e.g., mortality), although several host resistance tests have been used which incorporate continuous endpoints.

**Statistical analysis.** To examine the qualitative relationships between immune and host resistance tests, specificity, sensitivity, and concordance were determined as previously described (Luster *et al.*, 1992). To communicate the overall effect of a chemical on an endpoint, the practice has been to assign an ordinal-type classification. A general measure of immunotoxicity as well as measures for each assay can be assigned a "yes" or "no," denoting statistical significance for a result using the established criteria and comparisons can be made as to the ability of the immune test(s) to predict a yes/no change in the host resistance models. Sensitivity is, then, the probability of predicting an effect on the immune test given that the host resistance test was affected. This can be defined as  $a/(a + c)$  100%

**TABLE 1**  
**Host Resistance Models and Immune Tests Employed for Statistical Analyses<sup>a</sup>**

Challenge model	Endpoint monitored
<i>Listeria monocytogenes</i>	Morbidity <sup>b</sup>
<i>Streptococcus pneumoniae</i>	Morbidity
<i>Plasmodium yoelii</i>	Parasitemia
PYB6 Tumor	Subcutaneous tumors (tumor incidence)
B16F10 Melanoma	Lung tumor burden (nodules)
Influenza	Morbidity
<b>Immune tests</b>	
IgM plaque-forming cell (PFC) response to sheep red blood cells (SRBCs)	
Natural killer (NK) cell activity against YAC-1 tumor cells	
Mixed leukocyte response (MLR) to allogeneic leukocytes	
Cytotoxic T lymphocyte (CTL) response to P815 tumor cells	
T cell mitogenic response to concanavalin A (Con A)	
B cell mitogenic response to lipopolysaccharide (LPS)	
Delayed hypersensitivity response to keyhole limpet hemocyanin	
Surface marker expression including SIg, Thy 1.2, CD4, and CD8 <sup>c</sup>	
Peripheral leukocyte counts	
Spleen cellularity (nucleated cells)	
Thymus/body weight ratio	
Spleen/body weight ratio	

<sup>a</sup> For details see Luster *et al.*, 1988; Dean *et al.*, 1989.

<sup>b</sup> Morbidity is defined as "adequate indication that the animal may not survive until the next observation as judged by an experienced laboratory animal specialist" as described by Rao and Huff, 1990, and hence, considered categorical (dichotomous) data.

<sup>c</sup> Surface marker expression is used to define lymphocyte subpopulations: SIg<sup>+</sup>, B lymphocytes; Thy 1.2<sup>+</sup>, T lymphocytes; CD4, helper/inducer T lymphocytes; CD8, suppressor/cytotoxic T lymphocytes.

(converted from the probability scale 0–100) where, for example, *a* is the number of times that the immune and host resistance tests were affected and *c* is the number of times that the immune test was affected without affecting host resistance. Specificity represents the probability of correctly identifying chemicals that do not affect either parameter while concordance is the probability of making a correct decision regarding host resistance using the immune assay(s) as the sole parameter. Ordinal classifications for mutagenicity and carcinogenicity have been formed by the NTP, IARC, and other programs and agencies for many of the same chemicals (e.g., Tennant *et al.*, 1987). Fisher's exact test was used to assess the statistical significance of the concordances (Fleiss, 1981).

A second series of analyses was conducted to establish whether the host resistance and immune function relationships exhibit behavior which supported a "nonlinear" or "threshold-like" model for dose response. In this exercise, the "nonlinear" models arise from the risk assessment literature and not the statistical literature (Portier, 1990) and refers to models which have a slope of zero for dose-response at dose zero when dose is expressed on the arithmetic scale. Logistic regression modelling was used for assays in which the responses were portrayed as proportions (e.g., survival). For assays with more continuous responses (e.g., parasitemia or tumor counts), standard regression techniques were applied. In both cases, two different models were fit to the data; a linear model (Figs. 1A or 1B) and a linear-quadratic model (Figs. 1C or 1D). The linear-quadratic model allows for "threshold-like" dose-response; when the linear parameter is estimated to be zero, the slope of this model at dose zero is zero. In the standard regres-

sion framework, if the independent variable, *X*, is the immune function response expressed as a relative change from the control values, and *Y(X)* is the response from the assay, then the linear-quadratic model can be expressed as

$$Y(x) = \alpha_{1q} + \beta_{1q}X + \gamma_{1q}X^2 + E_{1q}, \quad (1)$$

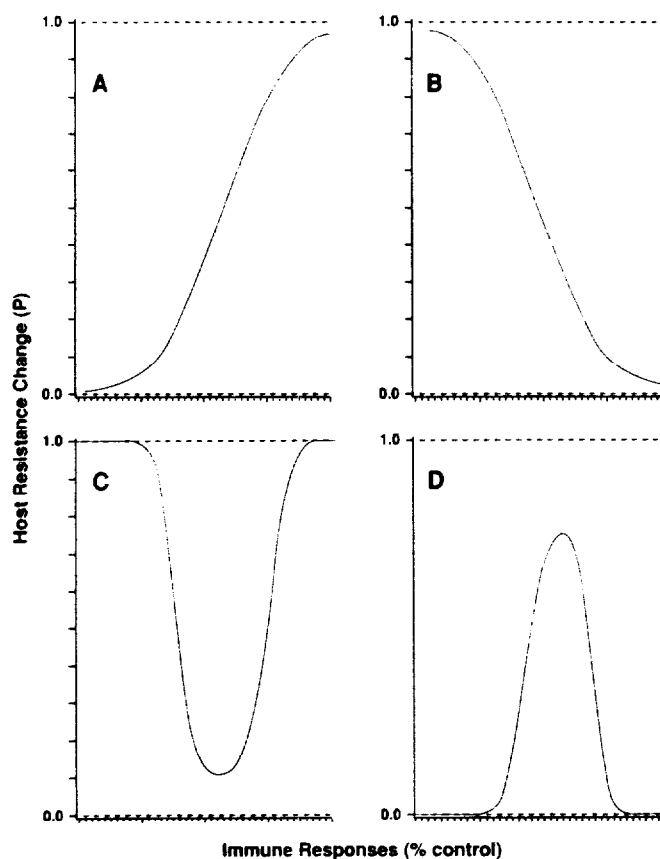
where  $\alpha$ ,  $\beta$ ,  $\gamma$  are parameters to be estimated and  $E_{1q}$  accounts for the random noise in the data. The linear model can be expressed as

$$Y(x) = \alpha_1 + \beta_1X + E_1, \quad (2)$$

where the parameters are similar to those in Equation 1. In the logistic regression framework, a similar model is used except the logit of the response proportion,  $P(x)$ , is written as

$$\ln \frac{P(x)}{1 - P(x)},$$

and  $P(x)$  is substituted for  $Y(x)$ . To test for the importance of sigmoidicity in the data, we tested the hypothesis that  $\gamma_{1q} = 0$ , and chose the linear-quadratic model if  $\gamma_{1q}$  was significantly different from 0 and chose the linear model if it was not. Instead of the usual  $p \leq 0.05$  significance level for testing,  $p \leq 0.10$  was employed, which is a more common level of testing for goodness of fit. Subjective visual methods were also used to assess curvature, but these results did not differ markedly from the objective, statistical approach presented here.



**FIG. 1.** Examples of logistic models exhibiting linear (sigmoidal; A, B) or linear-quadratic (threshold; C, D) relationships.

In order to better evaluate dose-response relationships for risk assessment for immunotoxicology, it was important to establish the change,  $\Delta(\pi)$ , in immune function responses which would result in a relative change of  $\pi$  in host resistance. This can be expressed mathematically as the value of  $\Delta(\pi)$  which satisfies the formula

$$\Pi = \frac{P[1 + \Delta(\pi)]}{1 - P(1)} \quad (3)$$

for the logistic regression model and

$$\Pi = \frac{Y[1 + \Delta(\pi)] - Y(1)}{Y(1)} \quad (4)$$

for the standard regression model. Since changes relative to the control responses are examined,  $Y(1)$  is used to denote the host resistance response at the control immune function response.

Given a change  $\pi$  in the probability of survival, it is possible to determine the associated value for  $\Delta(\pi)$ . For example, a linear model was sufficient to describe the relationship between *L. monocytogenes* resistance and changes in the antibody PFC response. For this model, we estimated  $\alpha_1 = 1.80$  and  $\beta_1 = 1.95$ . It then follows from (2) that

$$E[Y(x)] = 1.80 + 1.95x$$

where  $[Y(x)]$  is the expected (average) response. In this instance, the linear logistic model is

$$P(x) = \frac{e^{-1.80+1.95x}}{1 + e^{-1.80+1.95x}},$$

where  $x$  is the PFC response in terms of percentage control. It would follow that

$$P[\Delta(\pi) + 1] = \frac{e^{-1.80+1.95[\Delta(\pi)+1]}}{1 + e^{-1.80+1.95[\Delta(\pi)+1]}} \quad \text{and} \quad P(1) = \frac{e^{-1.80+1.95}}{1 + e^{-1.80+1.95}}.$$

Hence, if  $\pi = -0.05$  (e.g., if the change in probability of *Listeria* survival is a 5% decrease), then

$$\Delta(\pi) = \frac{\ln\left(\frac{-0.05 + e^{-1.80+1.95}}{1 + 0.05}\right) + 1.80 - 1.95}{1.95} = -0.048.$$

This implies that a 4.8% decrease from control in the PFC response would yield a 5% decrease from control in the probability of *Listeria* survival. The confidence intervals on these predictions were derived using the method outlined by Brand *et al.* (1973).

## RESULTS AND DISCUSSION

Despite overwhelming experimental and clinical evidence that increases in neoplastic and/or infectious disease occur in animals and individuals with secondary immunodeficiency (e.g., Austin *et al.*, 1989; Ehrke and Mihich, 1985), neither the most appropriate immune endpoints for predicting clinical disease nor the quantitative relationship between immune function changes and impairment of host defense are clearly defined. For example, it would be useful

to determine whether certain immune endpoints (or quantity of changes) predict certain outcomes (e.g., increased susceptibility to influenza and decreased antibody responses). A better understanding of these relationships would be particularly beneficial for risk assessment since changes in immune function are more readily quantifiable in populations at risk than are changes in the frequency or severity of clinical diseases, such as severity of infections. A particularly relevant question for risk estimation is whether increases in host susceptibility to challenge agents follow linear or "threshold-like" models as a function of increased immunosuppression. While terms such as immune reserve and immunological redundancy certainly are applicable when examining individual responses, it is unclear how these would apply in large populations. Since the potential outcomes from immunosuppression are increases in infections or neoplastic diseases, and there already exists a background incidence of these diseases in the population (e.g., Morbidity and Mortality Weekly Report, 1990), it would be helpful to determine the additional frequency of disease that would be associated with increasing changes in immune responses.

*Correlation between immune tests and altered host resistance.* Utilizing the NTP immunotoxicology rodent data base, as well as specifically designed data sets, we have attempted to address some of the above issues. Initially, analyses were conducted to establish the concordances between potential immunotoxic chemicals and altered host resistance. The values shown in Table 2 indicate that compounds which produce no evidence of changes in immune

TABLE 2  
Association of Each of the Host Resistance Models  
with the Immune Tests

Challenge agent	No. of tests	Frequency <sup>a</sup>		
		Specificity -/-	Sensitivity +/+	Concordance total
<i>L. monocytogenes</i>	34	100	52	65 <sup>c</sup>
PYB6 tumor	24	100	39	54
<i>S. pneumoniae</i>	19	100	38	58
B16F10 Melanoma	19	100	40	68
<i>Plasmodium yoelii</i>	11	100	38	55
Influenza	9	100	17	44
Any of the above <sup>b</sup>	46	100	68	78 <sup>c</sup>

<sup>a</sup> Frequencies are defined as specificity, the percentage of nonimmunotoxic chemicals yielding no effect on the host resistance models; sensitivity, the percentage of potential immunotoxic chemicals yielding a change in a host resistance model; concordance, percentage of qualitative agreement.

<sup>b</sup> Frequency calculated on any of the host resistance models. For B15F10 data, CPM rather than the number of nodules were used to determine significance.

<sup>c</sup> Agreement statistically significant at  $p < 0.05$  by Fisher's exact test.

response are not very likely to affect host resistance (100% specificity). However, compounds considered potential immunotoxicants have, at best, a 50% chance of affecting a host resistance test (sensitivity) which accounts for the relatively low concordance values. It should be noted, however, that for any compound tested, data from only two host resistance models were normally obtained. When this is minimized by using a host resistance classification based upon at least one positive host resistance test for the chemical studied, the sensitivity and subsequent concordance increased to 78%. The above values were based upon our described criteria of an immunotoxic chemical which admittedly is liberal. Thus, although a compound which may only cause a 10% decrease in CD4<sup>+</sup> cell numbers at the two highest dose levels tested may be considered positive by our criteria, it would have questionable clinical significance.

We then determined the ability of individual and pairs of immune tests to predict host resistance. For these studies, a compound was considered to alter host resistance if it resulted in a change ( $p < 0.05$ ) in one or more of the challenge models in the high-dose group (Appendix A). The concordance values for individual tests are given in boldface on

the diagonal of the matrix in Fig. 2. Although none of the individual tests were remarkable, many of the immune tests provided a relatively good association with changes in host resistance (i.e., >70%) with DHRs being the best. Several of the tests, such as leukocyte counts and lymphoproliferative response to LPS, were poor predictors for host resistance providing concordance values of approximately 50%. Figure 2 also provides concordance values for combinations of two tests (off-diagonal element) where a positive response was given when either test was positive. In almost all cases, concordance was increased from that obtained using individual tests. Pair-wise combinations which included either the PFC response, surface markers, or DHRs usually demonstrated higher concordances with the host resistance tests than other pairs.

In another analysis, which included chemical dose as an additional variable, the relationship between the immune and host resistance tests was examined for interdependence using Kendall's nonparametric rank correlation test (Fleiss, 1981). Figure 3 summarizes the results of this analysis for each immune assay and host resistance test where sufficient data were available for evaluation. As a cautionary note, the

	Plaque Forming Cells	NK Cell Activity	T Cell Mitogens	Mixed Leukocyte Response	Delayed Hypersensitivity	CTL	Surface Markers	Leukocyte Counts	Thymus/BW Ratio	Spleen/BW Ratio	Spleen Cellularity	LPS Response
Plaque Forming Cells	<b>73</b> (41)											
NK Cell Activity	77 (31)	<b>73</b> (33)										
T Cell Mitogens	78 (36)	77 (31)	<b>71</b> (41)									
Mixed Leukocyte Response	70 (30)	71 (28)	75 (32)	<b>68</b> (34)								
Delayed Hypersensitivity	73 (26)	79 (19)	81 (26)	81 (29)	<b>82</b> (28)							
CTL	100 (5)	83 (16)	75 (4)	80 (50)	—	<b>67</b> (6)						
Surface Markers	80 (20)	78 (18)	81 (21)	75 (20)	79 (14)	100 (2)	<b>71</b> (21)					
Leukocyte Counts	72 (25)	67 (21)	65 (26)	67 (24)	83 (18)	67 (3)	65 (17)	<b>56</b> (27)				
Thymus/BW Ratio	71 (35)	79 (28)	73 (33)	85 (27)	79 (24)	75 (4)	78 (18)	81 (26)	<b>76</b> (37)			
Spleen/BW Ratio	75 (36)	79 (29)	76 (34)	79 (28)	75 (24)	80 (5)	74 (19)	69 (26)	76 (37)	<b>74</b> (38)		
Spleen Cellularity	71 (31)	69 (26)	74 (27)	72 (25)	78 (19)	75 (4)	61 (18)	64 (22)	76 (29)	76 (29)	<b>71</b> (31)	
LPS Response	62 (34)	63 (27)	64 (36)	68 (28)	67 (24)	100 (3)	76 (17)	67 (24)	74 (31)	66 (32)	58 (24)	<b>54</b> (37)

FIG. 2. Individual and pairwise concordance to establish predictability of individual immune tests for any host resistance change. Values are presented as percentage concordance with individual concordance values shown in boldface on the diagonal of the matrix and combinations using two tests on the off-diagonal element. Individual chemical immune test results have been reported previously (Luster *et al.*, 1992). Individual chemical host resistance tests can be found in Appendix A.

	Plaque Forming Cells	LPS Response	NK Cell Activity	Con A Response	Mixed Leukocyte Response	Delayed Hypersensitivity	Cytotoxic T Lymphocyte	30 @ Surface Marker	Thy 1.2 @ Surface Marker
<i>L. monocytogenes</i>	16* (81)	00 (59)	20* (58)	24** (63)	12 (51)	15 (45)	40* (9)	32* (28)	19 (29)
<i>S. pneumoniae</i>	25* (45)	04 (42)	14 (36)	09 (45)	20 (45)	09 (37)	—	18 (32)	21 (32)
<i>P. yoelii</i>	32* (27)	06 (22)	00 (22)	12 (22)	06 (16)	51* (13)	—	38* (13)	04 (13)
Influenza	22 (21)	13 (19)	05 (18)	12 (20)	14 (22)	36* (17)	—	—	—
PYB6 sarcoma	05 (33)	00 (30)	50** (31)	26* (39)	42** (37)	11 (30)	54* (9)	12 (18)	43** (21)
B16F10 melanoma	06 (41)	04 (40)	16 (37)	37** (43)	15 (42)	34* (27)	—	28* (34)	12 (34)

FIG. 3. Correlation coefficients between host susceptibility and immune function changes as determined by Kendall's correlation. The number in parentheses represents the number of data points (chemicals plus dose levels) used to derive the values, \* $p < 0.05$ , \*\* $p < 0.01$ .

significance given to the correlation values is dependent upon the number of tests conducted for each of the comparisons. In most cases, multiple immune effects were associated with a decrease in a specific challenge model, and the changes in immune responses were generally consistent with the immune mechanisms associated with resistance to that particular agent. For example, increased susceptibility to PYB6 tumor cell challenge was associated with a decrease in NK cell activity and cell-mediated immune responses (i.e., MLR, CTL, Thy 1.2<sup>+</sup> numbers). A notable exception to this was a lack of association between NK cell activity and resistance to B16F10 melanomas. It was also apparent that a loss in the ability to generate normal antibody responses correlated with increased susceptibility to *L. monocytogenes*, *S. pneumoniae*, or *Plasmodium yoelii*. Decreases in NK cell activity correlated only with altered resistance to *L. monocytogenes* or PYB6 tumor formation.

Taken together, these data suggest that a good correlation exists between the immune assays and the host resistance tests in that host resistance changes occur only when immune changes are present. However, immune changes can arise in the absence of alterations in host resistance and this implies that either the immune tests are more sensitive than host resistance or that certain immune tests are predictive for only certain host resistance tests. If the latter is true, then increasing the number of host resistance tests used to evaluate a chemical should increase the concordance between the two functions, which was suggested from our analysis. These data also indicate that some immune tests are more predictive of a change in host resistance than others, but no single test is exceptionally predictive. It is conceivable, however, that either a large change in one immune parameter or lesser changes in multiple immune parameters may be sufficient to alter a specific challenge model. Although it is unlikely that a single immune test can be used to predict clinical disease, immune function/host resistance associa-

tions are not totally random. For instance, studies in humans with primary and secondary immunodeficiency have shown that individuals with deficient antibody responses are susceptible to acute pyrogenic infections such as *Pseudomonas*, *Staphylococci*, and *Streptococci*. Also, individuals with defects in cell-mediated immunity are prone to develop generalized infection from intracellular pathogens and neoplastic diseases. General associations of this type were also observed in the present studies employing Kendall's correlation.

*The influence of dose of the challenge agent.* A major purpose for undertaking these analyses was to characterize the quantitative relationship(s) between the various immune and host resistance tests. Two variables which could potentially confound this relationship are the virulence and amount of the infectious agent. These remain a constant for most individual experimental studies, but may vary be-

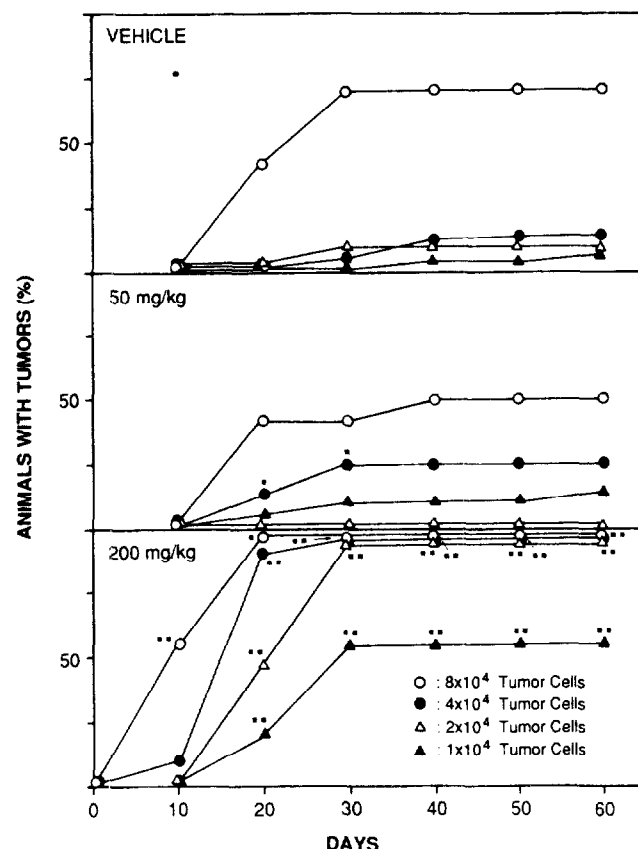


FIG. 4. Effects of cyclophosphamide on PYB6 tumor formation as a function of tumor cell challenge. Groups of mice were administered a single ip injection of saline, 50 or 200 mg/kg cyclophosphamide, followed 48 hr later by subcutaneous injection of either  $1 \times 10^4$  ( $\blacktriangle$ ),  $2 \times 10^4$  ( $\triangle$ ),  $4 \times 10^4$  ( $\bullet$ ), or  $8 \times 10^4$  ( $\circ$ ) PYB6 tumor cells. The data are presented as the percentage of animals that develop palpable tumors within 60 days. Each point represents 20 mice. \* $p < 0.05$  or \*\* $p < 0.01$  vs vehicle-treated group by Fisher's exact test at same time point given equal numbers of tumor cells by Fisher's exact test.

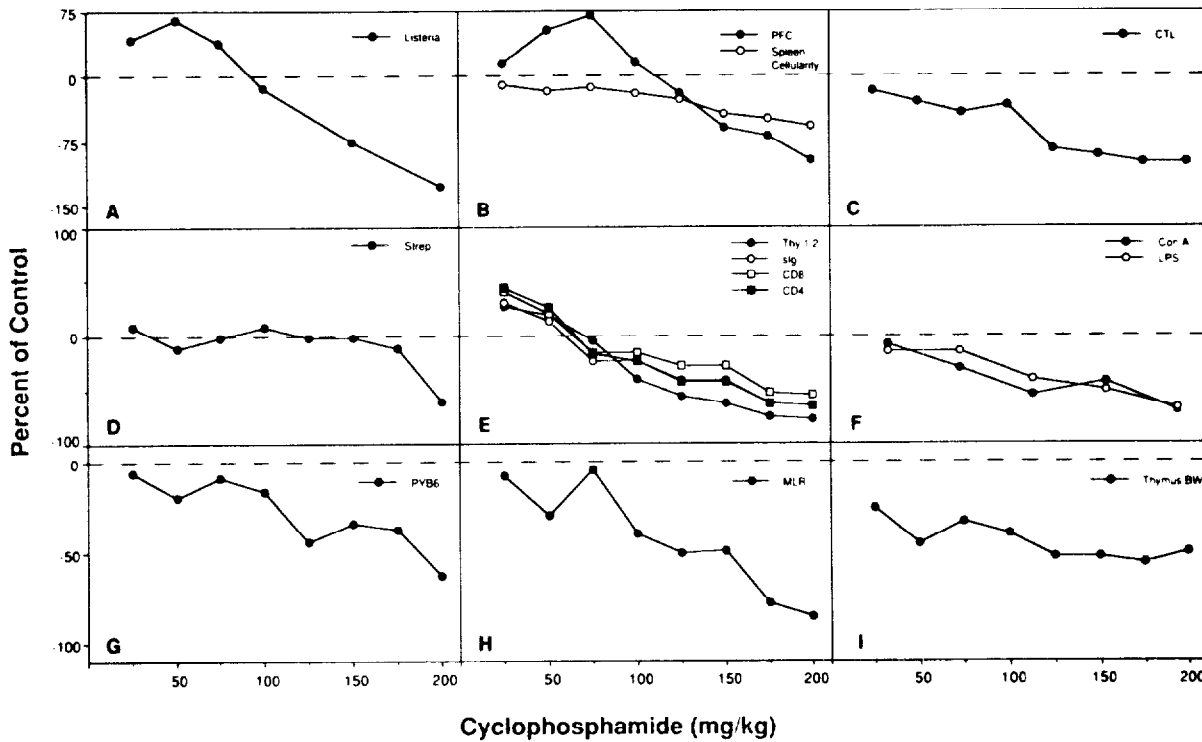


FIG. 5. Effects of cyclophosphamide on immune and host resistance parameters. Female B6C3F1 mice were injected with a single ip injection of cyclophosphamide at doses ranging from 0 to 200 mg/kg and immune assessment was initiated 48 hr later as described under Materials and Methods. (A) *L. monocytogenes* morbidity ( $n = 100/\text{group}$ ); (B) PFC response to SRBCs or spleen cellularity ( $n = 19/\text{group}$ ); (C) CTL response ( $n = 19/\text{group}$ ); (D) *S. pneumoniae* morbidity ( $n = 10/\text{group}$ ); (E) spleen lymphocytes expressing Thy 1.2, Slg, CD4, or CD8 ( $n = 19/\text{group}$ ); (F) lymphoproliferative responses to Con A or LPS ( $n = 10/\text{group}$ ); (G) tumor development in response to PYB6 tumor cell transplant ( $n = 20/\text{group}$ ); (H) mixed leukocyte response ( $n = 10/\text{group}$ ); (I) thymus/body weight ratios ( $n = 19/\text{group}$ ).

tween experiments as well as in the human population. For example, in the general population one can assume that an infectious disease such as influenza may develop in any individual, independent of their immune capacity or prior immunization, provided that the virulence or quantity of the challenging agent is sufficient to overwhelm the defensive capacities. This is illustrated experimentally in Fig. 4 where groups of mice pretreated with either vehicle (saline), 50 mg/kg cyclophosphamide (inducing minimal immunosuppression), or 200 mg/kg cyclophosphamide (inducing severe immunosuppression) were administered various

numbers of PYB6 tumor cells. Even vehicle-treated mice developed a high frequency of tumors provided the challenge was sufficiently high (i.e.,  $8 \times 10^4$  tumor cells). In contrast, severely immunosuppressed mice (high-dose cyclophosphamide) developed an increase in tumor frequencies at all PYB6 tumor cell challenge levels. The low-dose cyclophosphamide-treated groups were of particular interest where evidence of increased susceptibility appeared but only as a function of the tumor cell concentration. Assuming a large enough population is exposed and variability exists in the challenge dose, these data imply that even

TABLE 3  
Most Appropriate Relationships Describing the Host Resistance and Immune Tests

Host resistance test	Immune test									
	PFC	CTL	MLR	Con A	LPS	slg <sup>+</sup>	Thy 1.2 <sup>+</sup>	CD4 <sup>+</sup>	CD8 <sup>+</sup>	Thy/BW
<i>L. monocytogenes</i>	L	L-Q	L-Q	L	L	L-Q	L-Q	L-Q	L	L-Q
PYB6 Tumor	L	L	L	L	L	L-Q	L	L	L	L
<i>S. pneumoniae</i>	L-Q	L	L-Q	L	L	L-Q	L	L	L	N

Note. Abbreviations used: PFC, plaque-forming cells/ $10^6$  nucleated cells; Slg<sup>+</sup>, Thy 1.2<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, cell surface antigens for B, T, helper/inducer, cytotoxic/suppressor splenic lymphocytes; MLR, mixed leukocyte response; CTL, cytotoxic T lymphocytes; Con A, concanavalin A, LPS, lipopolysaccharide; Thy/BW, thymus/body weight ratios; L, linear parameter is needed; L-Q, linear-quadratic is needed; N, neither model is appropriate.

small changes in immune function could increase the likelihood of disease. There was no treatment-related affect on time to tumor appearance.

*Development of models to establish quantitative relationships between immune and host resistance tests.* In order to develop appropriate mathematical models to assess the relationship between the immune and host resistance tests, it was necessary to analyze a data set in which immunotoxicity could be examined over a wide dose range for a chemical agent. This was not possible using routine studies which employed, at most, three chemical dose levels. Therefore, a study was designed using the broad-spectrum immunotoxic compound, cyclophosphamide (see Materials and Methods and Fig. 5 legend for details). While all of the immune parameters examined showed a significant dose-related decrease, several parameters, including *Listeria* susceptibility, the antibody PFC response, and, to some extent, surface marker expression, were enhanced at low doses prior to

inhibition. This was not unexpected since a number of reports have shown that immune stimulation occurs at low dose levels of cyclophosphamide and has been attributed to the greater sensitivity of T-suppressor cells to cyclophosphamide compared to other immune cells (Turk and Parker, 1982).

Using either logistic regression or standard regression, linear and linear-quadratic models (see Fig. 1) were fit to these data to establish which model most accurately describes the relationship between each of the host resistance and immune function assays. The results of these analyses utilizing the cyclophosphamide data set are summarized in Table 3. Most of the immune-host resistance relationships appeared to approximate a linear model although certain immune tests, such as B cell numbers and streptococcal challenge have a significant degree of nonlinearity. Table 4 provides the parameter estimates for the most appropriate model for each relationship studied and were used to develop the values shown in Table 3.

TABLE 4  
Parameter Estimates for the Host Resistance Models Derived from the Cyclophosphamide Data Set

Host resistance assay	Immune test	Intercept parameter ( $\alpha$ )	Linear parameter ( $\beta$ )	Quadratic parameter ( $\gamma$ )	Fit of the model to the 18 Chemical data set <sup>a</sup>
<i>L. monocytogenes</i>	CD4 <sup>+</sup> <sup>b</sup>	-7.448	17.2189	-8.7348	$p = 0.40$
	CD8 <sup>+</sup>	-2.0395	2.718	—	
	Thy 1.2 <sup>+</sup>	-5.5497	12.1596	-5.6226	
	slg <sup>+</sup>	-3.3179	7.8315	-3.4658	
	Con A	-2.5639	2.7903	—	
	LPS	-3.4056	3.7609	—	
	PFC	-1.7967	1.9529	—	
	MLR	-8.4322	24.6678	-16.1513	
	CTL	-1.936	7.7635	-5.3727	
	Thymus/BW	-7.4597	20.0424	-12.2548	
PYB6 Tumor	CD4 <sup>+</sup>	-1.4539	2.6199	—	$p = 0.40$ $p = 0.96$
	CD8 <sup>+</sup>	-1.7390	2.7162	—	
	Thy 1.2 <sup>+</sup>	-1.3414	2.4458	—	
	slg <sup>+</sup>	-2.2451	7.3977	-3.8174	
	Con A	-2.0113	3.2686	—	
	LPS	-1.9064	2.9263	—	
	PFC	-0.6298	1.1861	—	
	MLR	-1.7328	4.506	-1.0016	
	CTL	0.0188	1.287	0.1625	
	Thymus/BW	-2.4051	3.9576	—	
<i>S. pneumoniae</i>	CD4 <sup>+</sup>	-0.8621	3.8149	—	
	CD8 <sup>+</sup>	-1.6262	4.4507	—	
	Thy 1.2 <sup>+</sup>	-0.8376	3.7934	—	
	slg <sup>+</sup>	-2.3966	13.5029	-7.9517	
	Con A	-1.9401	5.8436	—	
	LPS	-2.1769	5.9074	—	
	PFC	-0.7780	7.5494	-3.6261	
	MLR	-2.6186	14.9272	-10.2	
	CTL	2.0014	2.3256	-2.1773	
	Thymus/BW	—	—	—	

<sup>a</sup> Only  $p$  values greater than 0.01 are shown.

For each pair of host resistance and immune function tests that could be fit by one of the two logistic models, the mathematical function representing the relative difference was developed. For example, the model representing the relationship between *L. monocytogenes* resistance and CD4 cell number can be shown in mathematical terms as:

$$\Delta LM = \frac{e^{-(7.448+17.22\Delta CD4-8.73\Delta CD4^2)}}{1 + e^{-(7.448+17.22\Delta CD4-8.73\Delta CD4^2)}},$$

where  $\Delta LM$  represents the percentage change over control in *L. monocytogenes* resistance and  $\Delta CD4$  represents the percentage change over control in CD4 cell number. This function provided the opportunity to establish the quantitative relationship between each of the immune function and host resistance tests examined in the cyclophosphamide studies. The values presented in Table 5 show the percentage change in various immune tests that yielded either a 5, 10, or 20% decrease in the probability of altering host resistance along with confidence levels. For example, a 5% decrease in resistance to *L. monocytogenes*, relative to controls, would be reflected as a 4.7% decrease in the PFC response with confidence levels ranging between 3.7 and 6.5%. The probability values for the LPS response are not shown since they were essentially the same as those for Con A. As can be construed from these data, the actual estimated changes for most of the immune tests were similar between host resistance assays, but the confidence levels showed considerable variability. For example, the decrease in the PFC response resulting in a 5% decrease in host resistance was 4.7, 6.6, and 8.2% for *L. monocytogenes*, PYB6 tumors, and *S. pneumoniae*, respectively, while the upper confidence limits were 6.5, 19.2, and 36.5%, respectively.

**Quantitative relationships using the NTP data set.** A series of analyses was conducted to compare and apply the relationships established between the immune and host resistance tests in the cyclophosphamide data set to those obtained using the NTP data set. Rather than using all 51 compounds studied by the NTP, an 18 chemical subset was selected (indicated with asterisk in Appendix A). These chemicals were selected on the basis of completeness of the data set and represent a broad spectrum of immunotoxicants for which similar methods were employed. A likelihood ratio test was used to compare the parameters derived from the cyclophosphamide data to the best estimates from the combined 18 chemical data base. When analyzed as percentage of control response, the data sets were in relatively poor agreement. Of the 30 relationships evaluated, only 3 of the cyclophosphamide models studied fit the 18 chemical database (Table 4, last column). This illustrated that the relationships between host resistance and immune function observed for one compound or class of chemical agents may not be easily extrapolated to other agents. The components of the immune system have varying levels of interlocking and independent roles in host resistance and a

TABLE 5  
Changes in Immune Tests and Confidence Intervals Corresponding to Given Relative Differences in Host Resistance Utilizing the Cyclophosphamide Data Set

Host Resistance test	Immune test	Relative decreased host resistance (confidence interval)		
		5%	10%	20%
<i>L. monocytogenes</i>	CD4*	11.6 (3.0-21.4)	14.7 (5.7-23.6)	19.1 (10.3-27.2)
	CD8*	2.7 (1.9-4.9)	5.4 (3.7-9.7)	10.6 (7.1-19.1)
	Thy 1.2*	5.5 (2.7-13.2)	9.5 (5.2-17.0)	15.6 (9.5-23.0)
	SIg*	8.2 (3.1-14.5)	13.2 (6.0-18.8)	20.5 (11.2-25.7)
	Con A	3.2 (1.9-7.2)	6.4 (3.8-14.5)	12.8 (7.5-29.2)
	PFC*	4.7 (3.7-6.5)	9.5 (7.4-13.0)	18.9 (14.8-26.1)
	MLR	50.3 (37.8-56.9)	51.6 (39.3-58.2)	53.4 (42.1-60.8)
	CTL	67.8 (19.4-76.7)	69.7 (26.0-78.6)	73.1 (34.9-82.1)
	Thymus/BW	32.3 (6.4-100)	34.9 (12.3-100)	39.4 (22.6-100)
PYB6 Tumor	CD4*	2.5 (1.3-7.9)	4.8 (2.7-15.7)	9.4 (5.1-30.8)
	CD8*	2.5 (1.3-8.6)	4.9 (2.6-17.1)	9.6 (5.1-33.7)
	Thy 1.2*	2.7 (1.5-8.7)	5.3 (2.9-17.2)	10.3 (5.6-34.0)
	SIg*	16.5 (2.6-43.7)	20.4 (5.1-46.5)	25.9 (9.8-52.6)
	Con A	1.9 (1.0-7.8)	3.8 (2.0-15.5)	7.3 (3.8-30.7)
	PFC	6.6 (3.7-19.2)	13.0 (7.4-38.2)	25.6 (14.5-75.7)
	MLR	1.7 (1.0-4.3)	3.2 (1.9-8.4)	6.2 (3.7-16.3)
	CTL	9.6 (26-153)	18.9 (52-302)	36.6 (ND)*
	Thymus/BW	1.5 (0.7-39.7)	3.0 (1.3-7.9)	5.7 (ND)
<i>S. pneumoniae</i>	CD4*	1.4 (0.6-65.5)	2.6 (1.3-129)	5.1 (2.5-250)
	CD8*	1.2 (0.6-15.5)	2.3 (1.1-30.4)	4.4 (2.2-58.8)
	Thy 1.2*	1.4 (0.7-41.3)	2.7 (1.3-81.1)	5.1 (2.5-157)
	SIg*	48.6 (1.7-61.3)	49.8 (3.2-63.4)	52.0 (6.2-67.8)
	Con A	0.9 (0.5-6.8)	1.7 (0.9-13.3)	3.2 (1.7-25.7)
	PFC	8.2 (2.5-36.5)	11.6 (4.8-40.7)	16.2 (8.7-48.2)
	MLR	4.6 (0.7-88.1)	8.0 (1.5-89.0)	12.1 (2.9-90.8)
	CTL	90.4 (ND)	177 (ND)	340 (ND)
	Thymus/BW	1.4 (0.7-41.3)	2.7 (1.3-81.1)	5.1 (2.5-157)

\* Abbreviations used: PFC, plaque-forming cells/ $10^6$  nucleated cells; SIg\*, Thy 1.2\*, CD4\*, CD8\*, cell surface antigens for B, T, helper/inducer, cytotoxic/suppressor splenic lymphocytes; MLR, mixed leukocyte response; CTL, cytotoxic T lymphocytes; Con A, concanavalin A; Thymus/BW, thymus:body weight ratios.

\* ND, Not determinable due to divergence of parameters.

more mechanistically based approach for modeling this relationship will need to be undertaken. Computer models attempting to describe the relationships between the

various immune components have recently been undertaken (Celada and Seiden, 1992).

**Conclusions.** While these studies begin to provide insight into the qualitative and quantitative relationships between immune function and host resistance, there are a number of limitations in the analyses. For example, additional experimental studies, such as that conducted with cyclophosphamide using a large number of dose levels, will be required using other immunosuppressive regimens with different modes of action to provide additional clarification of the quantitative relationships. Particularly useful in such studies would be immunosuppressive compounds that produce unique "immunological profiles" such as those that may be selective for specific components of the immune system (e.g., cyclosporine). Another difficulty in establishing quantitative relationships stems from the redundancy of the immune system which is typified by the involvement of multiple immune components when responding to various foreign challenges. This is confounded in immunotoxicology studies due to the assumed nonspecificity for most chemical-immune interactions and will require a more mechanistically based approach for modeling. Thus, for some chemicals decreased antibody PFC responses paralleled increased susceptibility to *L. monocytogenes* infection while in other studies increased susceptibility occurred in the absence of any detectable effect on the PFC response, presumably affecting other cell components such as NK cell activity or CTL responses. It is also likely that an accumulation of "modest" effects on multiple immune components will be sufficient to alter host resistance. In addition to other chemical agents, additional analyses using other animal species and/or immune tests will also aid to further define the described relationships.

Taken together, the major observations from these analyses are the following: (1) A good correlation exists between changes in the immune tests and altered host resistance in

that there were no instances when host resistance was altered without affecting immune test(s). However, in many instances immune changes may be observed in the absence of detectable changes in host resistance. This can be interpreted to reflect that immune tests are, in general, more sensitive than the host resistance assays. (2) We could not identify any single immune test that can be considered highly predictive for altered host resistance. However, many of the assays, including DHRs, were good indicators and others, such as proliferative response to LPS and leukocyte counts, were relatively poor indicators for host resistance changes. Combining several immune tests (see Fig. 2) only slightly increases the ability to predict host resistance deficits. (3) Considering that there exists a "background" level of infectious diseases in the population, it is possible that any change in immune function would translate to a change in host resistance given that the population exposed is large enough. This can be shown experimentally (see Fig. 4) but would be difficult to demonstrate in a clinical study where "infectivity" (i.e., virulence and dose of infectious agent) cannot be controlled. (4) Logistic and standard regression modeling, using the cyclophosphamide data set indicated most immune function-host resistance relationships can be adequately described by a linear (in low dose) model (Table 3). For some pairs, this relationship could be confirmed using the 18 chemical data set. However, with most pairs it was not possible to establish the most appropriate model. (5) Finally, using the cyclophosphamide data set we have developed methods for modeling quantitative relationships between changes in selected immune tests and host resistance tests. It is impossible, at present, to determine how applicable these values will be for immunotoxic compounds with different immune profiles. However, as more analyses become available based upon unique immune profiles, our ability to estimate more accurately potential clinical effects from immunological tests should increase.

## APPENDIX A

### Immunotoxicology Data Base

Test article	CAS No.	Route	Length of exposure (days)	Host resistance assay <sup>a</sup>						Immunotoxicology classification <sup>d</sup>
				Listeria	PYB6 Tumors	B16F10 Tumors (CPM/lung)	<i>Plasmodium</i>	<i>Streptococcus</i>	Influenza	
Acetonitrile	75-05-8	INHAL	14	N	-	N	N	N	N	-
Aldicarb oxime*	116-06-3	GAV	14	-	N	-	N	-	N	-
Allyl isovalerate	2835-39-4	GAV	10 over 14	-	N	N	-	N	N	-
Arsine*	7784-42-1	INHAL	14	+	N	N	N	N	N	+
Azathioprine <sup>b</sup>	446-86-6	IP	22 over 30	N	N	N	N	N	N	+
Benzidine*	92-87-5	GAV	5	+	+	N	N	N	N	+
Benzo(a)pyrene*	50-32-8	SC	14	+	-	+	N	+	-	+
Benzo(e)pyrene	192-97-2	SC	14	-	N	-	N	-	-	-
<i>o</i> -Benzyl- <i>p</i> -chlorophenyl	120-32-1	SP	14	N	-	N	N	N	N	-
<i>t</i> -Butylhydroquinone	1948-33-0	GAV	14	N	N	-	N	-	N	-

**APPENDIX A—Continued**  
**Immunotoxicology Data Base**

Test article	CAS No.	Route	Length of exposure (days)	Host resistance assay <sup>a</sup>						Immunotoxicology classification <sup>d</sup>
				Listeria	PYB6 Tumors	B16F10 Tumors (CPM/lung)	Plasmodium	Streptococcus	Influenza	
Cadmium chloride	10108-64-2	WATER	90	—	—	N	N	N	N	+
Chemical mixture <sup>c</sup> *		WATER	14	—	—	N	+	N	N	+
4-Chloro- <i>o</i> -phenylenediamine <sup>*</sup>	95-83-0	GAV	14	N	—	—	N	—	N	—
2,4-Diaminotoluene <sup>*</sup>	95-80-7	GAV	14	—	—	—	N	+	N	+
Dideoxyadenosine <sup>*</sup>	4097-22-7	GAV	22 over 30	+	N	—	N	+	N	+
Diethylstilbestrol <sup>*</sup>	56-53-1	SC	14	+	+	—	N	—	N	+
Dimethylbenz( <i>a</i> )-anthracene <sup>*</sup>	57-97-6	SC	14	+	+	N	N	N	N	+
Dimethyl vinyl-chloride	513-37-1	GAV	14	+	—	N	N	—	—	+
Diphenylhydantoin	630-93-3	GAV	14	N	N	N	+	N	N	+
Ethyl carbamate <sup>*</sup>	51-79-6	IP	14	N	N	N	N	N	N	+
Ethylene dibromide	106-93-4	GAV	14	—	—	N	N	N	—	+
Formaldehyde	50-00-0	INHAL	21	—	—	—	N	N	N	—
Gallium arsenide <sup>*</sup>	1303-00-0	INTRA	14	+	—	+	—	—	N	+
Ginseng		IP	10	+	N	N	—	N	N	+
Hexachlorodibenzo- <i>p</i> -dioxin <sup>*</sup>	19408-74-3	GAV	14	+	N	+	N	—	N	+
Indomethacin	53-86-1	SC	5	+	—	N	N	N	N	+
Interferon- $\alpha$	9008-11-1	IP	10	N	N	N	N	N	+	+
Lithium carbonate	554-13-2	GAV	14	—	N	N	—	N	N	+
Methyl carbamate <sup>*</sup>	598-55-0	IP	14	N	N	N	N	N	N	—
Methyl isocyanate	624-83-9	INHAL	4	—	N	—	—	N	—	—
Nickel sulfate	7786-81-4	WATER	180	—	N	N	N	N	N	+
Nitrobenzene	96-95-3	GAV	14	—	N	N	N	N	N	+
Nitrofurazone	58-87-0	GAV	14	N	N	—	N	N	N	—
<i>N</i> -Nitrosodimethyl-amine <sup>*</sup>	62-75-9	IP	14	—	N	N	N	+	—	+
<i>m</i> -Nitrotoluene <sup>*</sup>	99-08-1	GAV	14	+	+	—	—	—	N	+
<i>p</i> -Nitrotoluene <sup>*</sup>	99-99-0	GAV	14	—	—	—	—	—	N	+
Ochratoxin A <sup>*</sup>	303-47-9	IP	8	N	+	N	N	N	N	+
Oxymetholone	434-07-1	FEED	14	—	N	N	N	N	N	—
Pentachlorophenol	87-86-5	FEED	14	N	N	N	N	—	N	+
Pentamidine isethionate <sup>*</sup>	140-64-7	SC	22 over 30	N	N	N	N	N	N	—
<i>o</i> -Phenylphenol	90-43-7	GAV	10 over 14	—	—	N	N	N	N	+
Phorbol myristate acetate	16561-29-8	SC	4 over 14	+	+	+	N	N	N	+
Ribavirin <sup>*</sup>	36791-04-5	GAV	14	N	N	N	N	N	N	+
2,3,7,8-Tetrachloro-dibenzo- <i>p</i> -dioxin	1746-01-6	GAV	14	N	N	N	+	N	N	+
Tetraethyl lead	78-00-2	GAV	14	N	N	N	N	—	N	+
Tetrahydrocannabinol <sup>*</sup>	1972-08-3	GAV	30	—	N	—	N	—	N	—
4,4-Thiobis(6- <i>T</i> -butyl- <i>M</i> -cresol) <sup>*</sup>	96-69-5	GAV	14	—	+	—	N	+	N	+
Toluene	108-88-3	GAV	14	—	—	—	—	—	N	—
Tris(2,3-dichloropropyl)phosphate	78-43-3	SC	3	—	—	N	N	N	N	—
Vanadium pentoxide	1314-62-1	INHAL	14	N	N	N	N	N	—	—
4-Vinyl-1-cyclohexene diepoxide	106-87-6	SP	5	—	—	N	N	N	—	+

Note. ABBREVIATIONS: INHAL, inhalation; GAV, gavage; IP, intraperitoneal; SC, subcutaneous; WATER, drinking water; FEED, feed; INTRA, intratracheal installation; SP, dermal.

\* Included in the 18 chemical data set.

<sup>a</sup> Positive (+), negative (—), or not done (N) as defined under Materials and Methods.

<sup>b</sup> Represents compounds in which no host resistance tests were conducted and, thus, not included in the Table 2 analyses.

<sup>c</sup> The mixture consisted of 25 common groundwater contaminants frequently found near toxic waste dumps as determined by EPA surveys. For details see Germolec *et al.* (1989).

<sup>d</sup> From Luster *et al.*, 1992.

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## EFFECTS OF TOLMETIN SODIUM DIHYDRATE ON NORMAL AND POSTSURGICAL PERITONEAL CELL FUNCTION

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**Abstract** — Recent studies utilizing intraperitoneal (i.p.) administration of NSAIDs to rabbits after surgical injury to the parietal peritoneum demonstrated macrophage involvement. NSAIDs are known to inhibit the metabolism of arachidonic acid to prostaglandins, which in turn modulate a variety of macrophage functions. Studies presented here examine the effects of tolmetin administration on peritoneal resident and post-surgical leukocyte functions, such as phagocytosis, the release of superoxide anion and tumoricidal activity. Rats, either non-surgical or following peritoneal surgery, were injected i.p. with tolmetin. At various times after treatment, the rats were sacrificed and peritoneal cells collected by lavage. The phagocytic capability of peritoneal leukocytes was transiently decreased 5–7 days after the administration of tolmetin to normal animals. However, administration of tolmetin during surgery extended the length of time that phagocytic capability was enhanced. In non-surgical controls, there was an elevation in superoxide anion release and tumoricidal activity 24 h after tolmetin administration. Superoxide anion release was suppressed at days 5 and 7 after treatment, but returned to control levels by day 14. Intraoperative administration of tolmetin significantly elevated superoxide anion release at days 3 and 5, phagocytosis at days 7 and 14 and tumoricidal activity at day 3. These studies suggest that compounds which suppress prostaglandin synthesis can modulate the function of resident and post-surgical peritoneal cells.

Recent studies indicate that perioperative administration of nonsteroidal anti-inflammatory drugs (NSAIDs), including ibuprofen, can reduce adhesion formation after peritoneal surgery (Nishimura, Nakamura & diZerega, 1983, 1984). The formation of adhesions is a major cause of infertility and post-operative pain in women. Therefore, a drug which would reduce the severity or even prevent post-surgical adhesion formation would be of clinical value (Ellis, 1971). However, before a drug can be utilized in this clinical setting, potential toxicologic manifestation must be determined. NSAIDs are known to inhibit the cyclooxygenase pathway responsible for the metabolism of arachidonic acid to prostaglandins (Vane, 1971; Flower, Gryglewski, Herbaczynska-Cedro & Vane, 1972). Prostaglandins mediate events which occur during the generation of inflammation including leukocyte infiltration, edema formation and endothelial cell pro-coagulant activities (Randall, Eakins & Higgs, 1980). A decrease in prostaglandin synthesis could, in turn, lead to a decrease in these inflammatory parameters (Randall *et al.*, 1980). If

events mediated by prostaglandins are necessary for wound healing and defense against infection, then administration of a drug which prevents prostaglandin synthesis might interfere with these processes. Therefore, the effects of acute intraperitoneal (i.p.) administration of tolmetin on immune parameters involved in the clearance of damaged tissue and infection were studied. These include phagocytosis of opsonized particles and quantitation of the respiratory burst potential of peritoneal cells through superoxide anion release. In addition, since macrophages activated for microbiocidal activities are also tumoricidal, the ability of peritoneal exudate cells to cytolyse radiolabelled tumor cells was also examined.

### EXPERIMENTAL PROCEDURES

#### *Animals*

Female rats were obtained from Simonson (Gilroy, CA) and housed in metal cage racks. The animals were given food and water *ad libitum* and

placed on a 12 h light/dark cycle. In order to examine the effects of tolmetin sodium dihydrate on the peritoneal cells of normal animals, 1 ml of saline containing 0, 0.3, 1.0, 30.0, 10.0 or 30.0 mg tolmetin (McNeil Laboratories, Spring House, PA) was injected i.p. at day 0. At various times after administration, the animals were sacrificed by CO<sub>2</sub> asphyxiation to minimize bleeding into the peritoneum which frequently occurs after cervical dislocation. Following sacrifice, cells were obtained by peritoneal lavage as described below.

To examine the effects of tolmetin on peritoneal exudate cells after surgery, animals underwent peritoneal trauma. The animals were anesthetized by intramuscular injection of 0.15 ml ketamine-acepromazine (Western Medical Supply, Arcadia, CA), then underwent a midline laparotomy followed by abrasion of the parietal peritoneum along the lateral aspect of the abdomen with sterile gauze until punctate bleeding occurred. The peritoneum and abdominal musculature were then closed with one layer of 4-0 Ethilon monofilament sutures (Ethicon, Somerville, NJ). Drug was then injected through the first layer of sutures and the skin closed with a second layer of continuous sutures. At various times after surgery the rats were sacrificed and peritoneal exudate cells collected by peritoneal lavage.

#### *Compound*

Tolmetin sodium dihydrate was a gift from McNeil Laboratories (Spring House, PA). Tolmetin powder was readily soluble in physiologic saline at a concentration of 30 mg/ml. The solutions were prepared within 1 h of injection and passed through a 0.22  $\mu$ M filter just prior to injection.

#### *Monolayer preparation*

After sacrifice, the abdominal muscle was exposed, drawn away from the abdominal organs and 10 ml of ice cold, sterile phosphate-buffered saline injected via an 18 gauge needle. The abdomen was then massaged for approximately 1 min and 7-9 ml of lavage fluid withdrawn. Any samples containing blood or signs of infection were omitted. The number of viable nucleated cells was then determined by the enumeration of cells excluding the vital dye, trypan blue, in a hemacytometer field. Cells were pelleted by centrifugation at 1100 rotations/min for 10 min and then resuspended at  $2 \times 10^6$  nucleated cells/ml in RPMI 1640 supplemented with 4 mM glutamine, 10% heat-inactivated fetal calf serum and antibiotics (penicillin, streptomycin and fungizone) (Gibco, Grand Island,

NY). The cells were plated at the concentrations described in the assays below and allowed to adhere for 2 h at 37°C in 5% CO<sub>2</sub>, 95% air. At the end of this culture period, the cells were washed three times with pre-warmed phosphate buffered saline (PBS) to remove non-adherent cells. Each assay described below was performed on cells from individual animals.

#### *Phagocytosis*

Peritoneal cells,  $2 \times 10^6$ , from control and treated rats were allowed to adhere to glass coverslips (22  $\times$  22 mm) for 2 h at 37°C in 5% CO<sub>2</sub>, 95% air. After this incubation, the coverslips were washed thoroughly with PBS to remove non-adherent cells. During this time, heat-killed (100°C, 60 min) yeast (Sigma, St Louis, MO) were opsonized for 90 min with 20% adult rat serum to promote phagocytosis, washed three times and resuspended at a final concentration of  $2 \times 10^9$ /ml. An aliquot (100  $\mu$ l) of opsonized yeast was then added to the coverslip and this mixture was allowed to incubate for 15 min at 37°C in 5% CO<sub>2</sub>, 95% air. At the end of this time, the coverslips were washed thoroughly with PBS, inverted onto a glass slide and sealed with clear nail polish to prevent dehydration. The samples were immediately evaluated microscopically to determine (a) the percentage of adherent cells which contained yeast; and (b) the number of yeast cells ingested per cell. Care was taken to insure the number of yeast cells that were enumerated per cell were actually ingested and not just attached to the surface of the cells. At least 100 adherent cells were evaluated per sample and the percentage and average number of yeast cells ingested per peritoneal cell was calculated (Boxer, 1981).

#### *Superoxide anion production*

A 0.3 ml aliquot of peritoneal cells ( $2 \times 10^6$ /ml) was cultured in duplicate for 2 h in a 16 mm well of a Falcon 24-well plate. The macrophage monolayers were washed free of non-adherent cells and over-laid with a solution of Krebs's Ringers phosphate dextrose solution (consisting of physiologic saline, 0.04% KCl, 0.016% CaCl<sub>2</sub>, 0.032% MgSO<sub>4</sub>, 3 mM NaH<sub>2</sub>PO<sub>4</sub>, 13.6 mM Na<sub>2</sub>HPO<sub>4</sub> and 2.2mg/ml dextrose) containing 80  $\mu$ M ferricytochrome c (Sigma, St Louis, MO), 0.5  $\mu$ g/ml phorbol 12-myristate 13-acetate (Sigma, St Louis, MO) and, where appropriate, 100  $\mu$ g/ml superoxide dismutase (Sigma, St Louis, MO). The cells were then incubated for 1 h at 37°C, after which time superoxide dismutase-inhibitable cytochrome c reduction

was monitored using a spectrophotometer at 550 nm. Since superoxide dismutase scavenges superoxide anion before the latter can reduce cytochrome c, this assay is a reliable measurement of superoxide anion production (Curnutte & Babier, 1974).

Culture wells without macrophages were included as controls for each combination of reagents. The absorbance of this blank was subtracted from the absorbance of cytochrome c incubated with cells. There is a 1:1 stoichiometry between the amount of superoxide anion produced and the amount of cytochrome c reduced during this assay. Therefore, the extinction coefficient for cytochrome c ( $2.1 \times 10^4$ /M/cm) was used to quantitate the release of superoxide anion (Johnston, Godzik & Cohn, 1978).

#### Protein determination

To correct for the number of peritoneal cells adherent to the culture wells, the amount of protein remaining in the tissue culture wells used in the assay was determined. At the conclusion of the superoxide anion release assays, the plates were rinsed three times with PBS and allowed to dry overnight. At this time 200  $\mu$ l of water (double distilled, H<sub>2</sub>O) was added to each well to insure equal resuspension volume for each sample. To each well, 2 ml of BCA Protein Assay Reagent (Pierce Chemical Company, Rockford, IL) was added. This mixture was allowed to incubate for 1 h at 37°C and the absorbance at 562 nm was determined. Bovine serum albumin was used to derive a standard curve to determine the amount of protein present in each well. The number of nmoles of superoxide anion released was then converted to nmoles superoxide anion/mg cell protein.

#### Tumoricidal activity

An SV-40-transformed line of mouse kidney cells (TCMK-1) maintained in DMEM with 10% FCS was used as the target cell. The tumor cells were radiolabeled with 1.0  $\mu$ Ci/ml <sup>3</sup>H-thymidine for 18–24 h, removed from the culture flask by trypsinization, washed to remove radiolabel that was not cell-associated, and then resuspended in RPMI with 10% serum for addition to macrophage monolayers. An aliquot, 1.3 ml of the macrophage suspension described above, was allowed to adhere to duplicate 16 mm wells in Falcon 24-well plates. The monolayers were washed and overlaid with a suspension of labeled tumor cells, such that the effector-to-target cell ratio was 8:1. A positive control for tumoricidal activity was used. This

control consisted of adherent resident peritoneal cells which were incubated for 16 h with 0.1  $\mu$ g/ml lipopolysaccharide and lymphokine-containing supernatant (50% medium from cultures of rat splenocytes stimulated for 48 h with 5  $\mu$ g/ml Con A) before addition of radiolabelled tumor cells. The macrophage-tumor cell mixture was then incubated for 2 days at 37°C in 5% CO<sub>2</sub>. An aliquot of the supernatant was then removed from each culture dish, placed in a scintillation vial with fluor, and counted in a scintillation counter to determine the amount of label released from the tumor cells. Control cultures included tumor cells with resident (non-surgical) macrophages, and tumor cells alone (no macrophages, for the determination of spontaneous and total radiolabel release from the tumor cells). Cytotoxicity is expressed by the following formula:

$$[(E - S)/(T - S)] \times 100,$$

where *E* is the counts/min of label (experimental) released into the supernatant of macrophages cultured with tumor cells, *S* is the counts/min of spontaneous label release from the tumor cells cultured alone, and *T* is the counts/min of tumor cells lysed in the culture well by 0.5% sodium dodecyl sulfate (total release) (Bryant & Hill, 1982).

#### Statistics

All groups were analyzed by one-way analysis of variance followed by Duncan's new multiple range test. A *P* value  $\leq 0.01$  was considered significant.

## RESULTS

Since prostaglandins are thought to modulate leukocyte infiltration following inflammatory stimuli, the number of peritoneal cells harvested from control and surgical animals following acute tolmetin administration was examined (Table 1). In this instance, the doses of tolmetin given did not significantly alter the number of peritoneal macrophages harvested by lavage. However, microscopic examination of slides containing samples of peritoneal cells from each animal stained with Wright's differential stain indicated that the cell populations present in the peritoneum were altered after tolmetin administration (Fig. 1a). At days 1 or 3 following i.p. injection of 3 or 10 mg of tolmetin, there was an increase in the percentage of granulocytes in the peritoneal lavage. However, by 5 days after tolmetin administration, the ratio between peritoneal macrophages, granulocytes and

Table 1. Effects of tolmetin administration on the number of viable nucleated cells harvested

Dose*	Normal				
	Days following administration† (cell number $\times 10^6$ )				
	1	3	5	7	14
0 mg	14.6 $\pm$ 1.2	13.2 $\pm$ 2.8	19.8 $\pm$ 2.9	20.8 $\pm$ 2.9	13.7 $\pm$ 2.3
0.3 mg	15.6 $\pm$ 2.6	17.7 $\pm$ 1.9	ND	19.6 $\pm$ 0.6	12.8 $\pm$ 0.6
1.0 mg	17.3 $\pm$ 1.5	21.0 $\pm$ 1.4	22.4 $\pm$ 0.4	20.4 $\pm$ 3.1	13.1 $\pm$ 1.0
3.0 mg	13.7 $\pm$ 0.8	16.5 $\pm$ 1.9	16.4 $\pm$ 1.2	23.5 $\pm$ 3.4	13.5 $\pm$ 0.7
10.0 mg	13.3 $\pm$ 0.9	15.1 $\pm$ 2.4	18.9 $\pm$ 1.1	16.4 $\pm$ 3.1	12.2 $\pm$ 3.4
30.0 mg	15.8 $\pm$ 2.5	13.9 $\pm$ 0.7	13.5 $\pm$ 2.4	22.7 $\pm$ 4.6	12.2 $\pm$ 1.5

Dose	Surgical‡				
	Days following administration (cell number $\times 10^6$ )				
	1	3	5	7	14
0 mg	19.8 $\pm$ 2.7	24.9 $\pm$ 1.7	27.9 $\pm$ 5.3	15.1 $\pm$ 1.0	21.3 $\pm$ 2.4
0.3 mg	21.5 $\pm$ 1.9	21.5 $\pm$ 1.6	19.2 $\pm$ 1.7	17.7 $\pm$ 1.4	13.9 $\pm$ 1.3
1.0 mg	19.0 $\pm$ 2.9	20.0 $\pm$ 2.9	20.1 $\pm$ 3.2	14.7 $\pm$ 1.2	15.2 $\pm$ 1.8
3.0 mg	20.1 $\pm$ 2.5	24.4 $\pm$ 3.2	25.5 $\pm$ 3.1	15.4 $\pm$ 2.2	16.3 $\pm$ 0.9
10.0 mg	18.7 $\pm$ 3.2	27.0 $\pm$ 3.4	18.5 $\pm$ 2.1	12.9 $\pm$ 1.3	18.2 $\pm$ 1.5
30.0 mg	21.0 $\pm$ 1.9	16.8 $\pm$ 1.9	17.8 $\pm$ 1.9	14.8 $\pm$ 1.3	15.3 $\pm$ 1.4
Resident	15.0 $\pm$ 0.7	15.2 $\pm$ 1.1	15.6 $\pm$ 2.1	11.4 $\pm$ 0.3	12.1 $\pm$ 0.9

These data represent the mean and standard error of 7 animals.

\*Rats were injected i.p. with 1 ml of saline containing the indicated amount of tolmetin sodium dihydrate.

†The time between treatment of the animals and sacrifice of the animals.

‡Surgery consisted of a midline laparotomy followed by sidewall abrasion under ketamine anesthesia.

ND = not determined.

lymphocytes was similar for control and treated rats. Following surgery, there was an initial influx of neutrophils followed by a shift in the leukocyte populations to increases in the percentage of macrophages. However, at a dose of 3 mg or greater, tolmetin treatment prolonged the length of time for which neutrophils were present (Fig. 1b).

The effects of acute i.p. injection of tolmetin on peritoneal leukocyte function from both non-surgical controls and post-surgical rats were also examined. Phagocytosis of opsonized particles by macrophages and neutrophils constitutes an initial defense mechanism against infection. In addition, increases in phagocytic capabilities occur following inflammatory stimuli. Therefore, the effect of tolmetin on phagocytosis was examined. As can be seen in Figs 2a and 3a, the percentage of adherent peritoneal cells able to phagocytize (Fig. 2a) and the number of yeast ingested per adherent cell (Fig. 3a) were significantly, but transiently, suppressed at days 5 and 7 after administration of tolmetin. In addition, at day 1 after tolmetin treatment, there was a slight decrease in the number of yeast phagocytized per adherent peritoneal cell. However, at day 14 after tolmetin administration, there was a dose dependent increase in the number of yeast ingested

per cell (Fig. 3a) and the percentage of cells that were phagocytic (Fig. 2a).

Administration of tolmetin sodium dihydrate during surgery did not significantly affect the percentage of adherent cells able to phagocytose opsonized yeast until days 7 and 14 after treatment (Fig. 2b). Surgical trauma increased the number of opsonized yeast per adherent cell within 1 day and was maximal by day 5 (Fig. 3b). By post-surgical day 7, these parameters returned to resident levels. However, administration of tolmetin during surgery extended the length of time phagocytic capability was increased, reaching maximal values 7 days after surgery. The increase in the number of yeast ingested per adherent cell was dose dependent, achieving a maximum value at 3.0 mg tolmetin. At post-surgical day 14, phagocytic activity returned to control levels.

The doses of tolmetin, which elevated the numbers of granulocytes in the peritoneum, increased the level of superoxide anion released by peritoneal cells one day after tolmetin administration (Fig. 4a). By days 5 and 7, the amount of superoxide anion released in response to stimulation with PMA was suppressed. However, this suppression was transient and the ability of peritoneal cells from treated rats to generate superoxide anion was equal to control levels

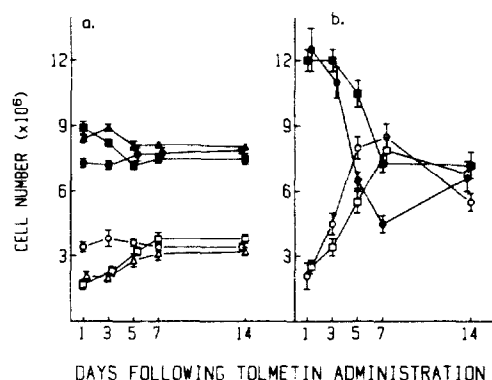


Fig. 1. Rats, either non-surgical (panel a) or surgical (panel b) were injected with tolmetin and sacrificed at various times after exposure. Following sacrifice, the peritoneal cells were harvested by lavage, suspended in RPMI 1640 and then cytospun onto microscope slides. The samples were stained with Wright's stain for differential counting and the relative number of granulocytes, macrophages and lymphocytes present within 10 random fields determined. Only the groups that were determined to be significantly different from control are presented for clarity. The groups are as follows: Panel a — control (●); control macrophages (○); 3 mg tolmetin granulocytes (■), 3 mg tolmetin macrophages (□); 10 mg tolmetin granulocytes (▲); 10 mg tolmetin macrophages (△); Panel b — control granulocytes (●); control macrophages (○); tolmetin treated granulocytes (■); tolmetin treated macrophages (□).

by day 14. Similarly, superoxide anion production by peritoneal cells was increased by surgical trauma, and tolmetin administration further increased superoxide anion release. Tolmetin administration prolonged the length of time between initiation of inflammation (caused by surgical trauma) and the influx of macrophages into the peritoneum. In surgical animals injected with saline, macrophages were the predominant leukocyte in the peritoneal exudate by post-operative days 4 and 5; in treated animals, this did not occur until day 7 (Fig. 1b). As with the non-surgical animals, increases in granulocyte numbers were paralleled by increases in superoxide anion release (Fig. 4b). In tolmetin-treated animals, superoxide anion production began a return to control levels by post-surgical day 7.

In an immune inflammatory response (that is, inflammation resulting in lymphocyte and lymphokine involvement), peritoneal leukocytes, particularly macrophages, exhibit tumoricidal activity. Tumoricidal activity is a measure of fully differentiated and activated macrophages (Hibbs, Taintor, Chapman & Weinberg, 1977; Hibbs,

Chapman & Weinberg, 1978). Following administration of tolmetin to non-surgical animals, there was an increase at days 1 and 3 in the ability of adherent peritoneal cells to lyse TCMK tumor cells in a 48 h assay (Fig. 5a). After surgery, peritoneal cells from rats injected with saline also demonstrated increased tumoricidal activity. Treatment with tolmetin, however, enhanced tumoricidal activity up to two-fold on days 1 and 3. By day 5, the level of tumoricidal activity exhibited by animals treated intraoperatively did not differ from saline treated surgical controls (Fig. 5b). The tumoricidal activity of the surgical animals peaked on day 7 and returned to resident levels by day 14.

In summary, acute administration of tolmetin after surgery elevated phagocytosis, superoxide anion and tumoricidal activity of peritoneal cells. The time of exposure and dose of tolmetin necessary to modulate immune function varied with the functional parameter examined.

## DISCUSSION

Since adhesions are a major source of post-operative pain and infertility after peritoneal surgery, drugs which reduce this process may be clinically useful. NSAIDs have been shown to inhibit cyclooxygenase enzyme activities which would, in turn, reduce prostaglandin synthesis (Tomlinson, Ringold, Qureshi & Forchielli, 1972; Ho & Esterman, 1974; Taylor & Salata, 1976). Prostaglandins were previously shown to mediate many inflammatory functions of macrophages and neutrophils (Higgs & Salmon, 1979). Macrophages and neutrophils are centrally involved in the initial clearance of bacteria and damaged tissue. Macrophages and neutrophils manifest increased capacities including phagocytosis of opsonized particles (Bianco, Griffin & Silverstein, 1975; Ratzan, Musher, Keusch & Weinstein, 1972) superoxide anion release (Johnston *et al.*, 1978; Nathan & Root, 1977) tumoricidal/microbiocidal (Hibbs *et al.*, 1978) activity following inflammatory and other stimuli. These enhanced functions, in turn, allow for the rapid clearance of infectious agents. Since many of these functions which are enhanced during inflammation may be regulated by prostaglandins, administration of a compound such as an NSAID, which inhibits prostaglandin synthesis (Taylor & Salata, 1976), many modulate these enhanced granulocytic cell functions, thereby altering the ability of a treated animal to clear infection.

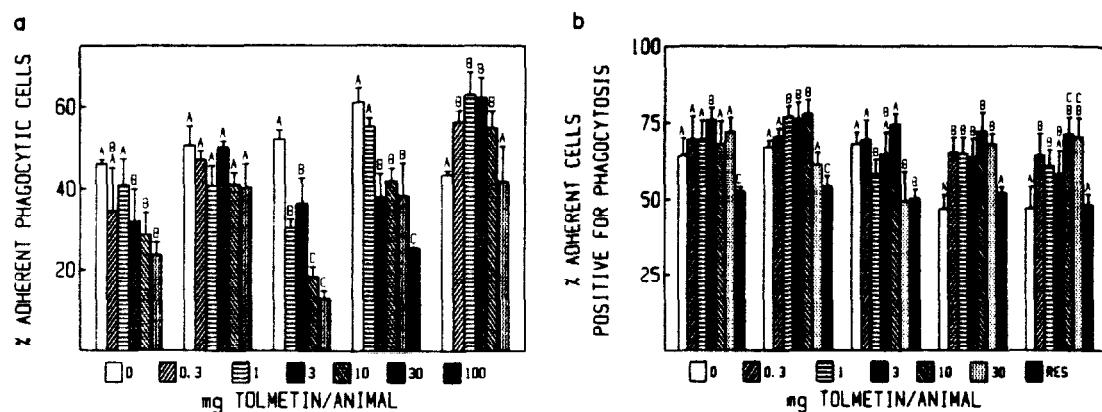


Fig. 2. In panel a, non-surgical animals were treated with various doses of tolmetin (listed on x-axis) and sacrificed at various times. In panel b, rats were treated with tolmetin following surgery and sacrificed at various times. Following sacrifice, the peritoneal cells were harvested by lavage and then adhered for 2 h to glass coverslips at 37°C. The coverslips were washed and opsonized yeast added to the coverslip for 20 min at 37°C. Following this incubation, the coverslip was washed, inverted and the percentage of adherent cells containing yeast determined. Results shown represent the mean and S.E. of five animals. The data were analyzed by one-way analysis of variance followed by Duncan's new multiple range test ( $\alpha = 0.01$ ). Bars with the same letter were considered statistically identical ( $P \leq 0.01$ ) by this analysis.

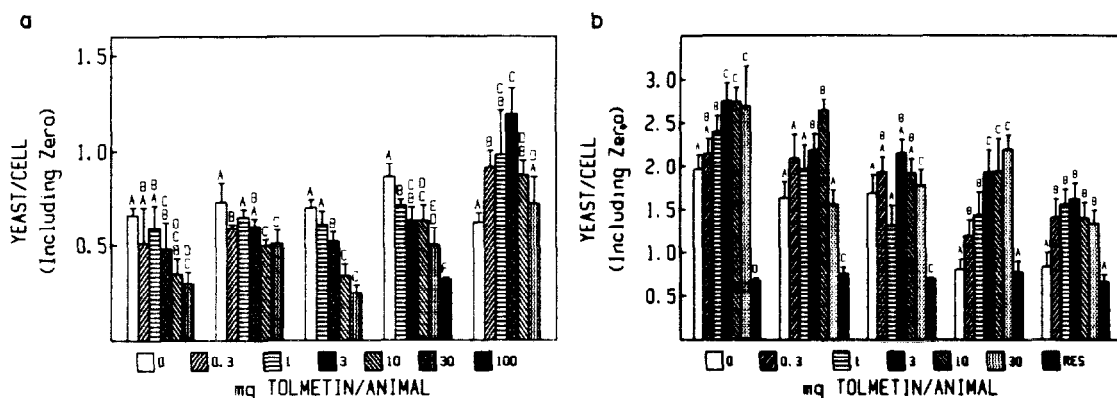


Fig. 3. The rats were treated as described in Fig. 2. The data represented in this figure are the number of opsonized yeast ingested per adherent cell. Results represent the mean and S.E. of five animals. The data were analyzed by one-way analysis of variance followed by Duncan's new multiple range test ( $\alpha = 0.01$ ). Bars with the same letter were considered statistically identical ( $P < 0.01$ ) by this analysis.

During peritoneal surgery there are many opportunities for the introduction of bacteria, therefore the effects of these drugs on capacities of peritoneal leukocytes, which are thought to be involved in the clearance of infection, are important. Acute administration of tolmetin to non-surgical animals transiently suppressed the release of superoxide anion and the ability of cells to phagocytize opsonized particles on days 5 and 7, but these responses returned to control levels by day 14. After surgery, administration of tolmetin significantly elevated superoxide anion release at days 3 and 5, phagocytic capability at days 7 and 14 and tumoricidal activity at day 3. Differential

staining and microscopic analysis revealed increases in neutrophil numbers at tolmetin doses of 3 and 10 mg. Although prostaglandins modulate neutrophil chemotaxis during inflammation, the regulation of homeostases in the normal peritoneum by prostaglandins is unknown. Prostaglandin synthesis and release were found to initially decrease during inflammation followed by a concomitant increase in neutral protease secretion by macrophages (Humes, Burger, Galavage, Kuehl, Wightman, Dahlgren, Davies & Bonney, 1980; Unkeless, Gordon & Reich, 1974; Werb & Gordon, 1975a, b). Taken together, these findings suggest that prostaglandin synthesis by macrophages is necessary

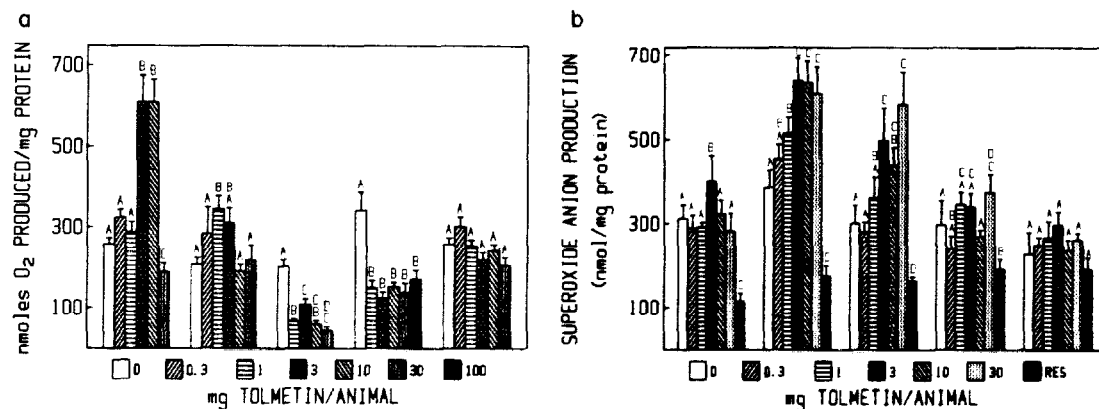


Fig. 4. The rats were treated as described in the legend of Fig. 2. Following sacrifice, the peritoneal cells were harvested, resuspended in RPMI 1640 and 10% FCS and 0.3 ml was placed in 16 mm wells of a 24-well plate for 2 h. At the end of this time, each well was washed three times and 1 ml of KRPD containing cytochrome c, PMA and, where appropriate, superoxide dismutase (as a blank) was added. These cultures were incubated for 1 h at 37°C and the absorbance at 550 nm determined. The nmoles of cytochrome c reduced per mg of protein was then calculated. Each dose and time point result shown represents the mean and S.E. of five animals. The data were analyzed by one-way analysis of variance followed by Duncan's new multiple range test ( $\alpha = 0.01$ ). Bars with the same letter were considered statistically identical ( $P \leq 0.01$ ) by this analysis.

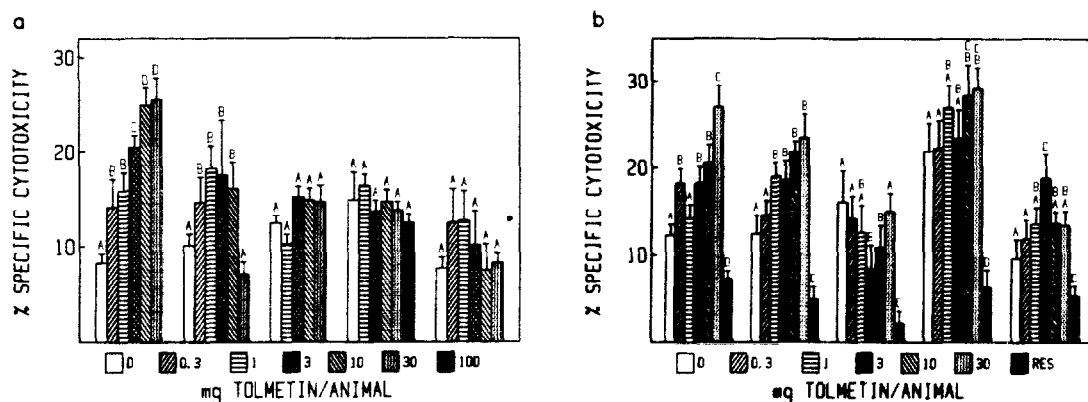


Fig. 5. The rats were treated as described in the legend of Fig. 2 and the peritoneal cells, 1.3 ml/well, were placed in 16 mm wells for 2 h. Positive controls for tumoricidal activity were generated by pre-incubation of resident peritoneal adherent cells with 0.1  $\mu\text{g}/\text{ml}$  lipopolysaccharide and lymphokine-containing supernatant. At the end of this time, each well was washed with PBS and  $2.0 \times 10^5$   $^3\text{H}$ -thymidine-labeled TCMK cells were added to each well and incubated at 37°C or 48 h. A sample of the supernatant was then harvested and the amount of  $^3\text{H}$ -thymidine released determined. The specific cytotoxicity for the positive control samples were between 48 and 65%. At all time points and doses, the positive controls were significantly different from all other groups. Each dose and time point result shown represents the mean and S.E. of five animals. The data were analyzed by one-way analysis of variance followed by Duncan's new multiple range test ( $\alpha = 0.01$ ). Bars with the same letter were considered statistically identical ( $P < 0.01$ ) by this analysis.

to maintain the resident differentiative state of the macrophage. If this is the case, inhibition of prostaglandin synthesis by a NSAID may provide the necessary signal(s) to initiate macrophage (and perhaps neutrophil) differentiation.

Increases in tumoricidal activity at days 1 and 3 cannot be explained by alterations in the types of

leukocytes present in the peritoneum. Tumoricidal activity of resident peritoneal macrophages activated with bacterial LPS is down-regulated by prostaglandins (Taffet & Russell, 1981; Schultz, Pavlielis, Styles & Chirigos, 1978; Taffet, Eurell & Russell, 1982). Lipopolysaccharide represents one stimulus which can generate inflammatory

macrophages. From data presented here, peritoneal surgery can also stimulate peritoneal cells; perhaps to an inflammatory state. Therefore, there are two stimuli present in this system, that is, surgical injury and inflammation and administration of tolmetin, which could prostaglandin synthesis. Interactions between these stimuli could, in turn, lead to the observed manifestation of tumoricidal activity.

At days 5 and 7 after tolmetin administration, phagocytic capacity and superoxide anion release were significantly suppressed. Since the sodium form of tolmetin is readily soluble in aqueous media and the peritoneal contents are rapidly cleared, these effects were probably due to secondary and not direct effects of the drug. If prostaglandin synthesis is partially responsible for suppression of resident peritoneal cell function, modification of the levels of this regulatory molecule may lead to a suppression of peritoneal cell function. Although this hypothesis is consistent with the observed results, there is no direct evidence to support this supposition.

Following surgery, there was an increase in the functional activity of peritoneal exudate cells as would be expected after an inflammatory stimulus. The phagocytic capability of adherent peritoneal leukocytes was significantly elevated which would allow for a more rapid removal of damaged tissue and any potential infectious agents. By post-surgical day 7, the activities of the peritoneal cells from control animals returned to resident cell levels. Administration of tolmetin lengthened the time following surgery during which peritoneal leukocyte

function was elevated. Microscopic examination of differentially stained samples from peritoneal lavage of control and treated rats indicated that the influx of macrophages into the peritoneum after surgery was delayed by tolmetin administration. In control and treated animals, the infiltration of macrophages into the peritoneum corresponded to the time point at which the early elevations in peritoneal leukocyte phagocytic and respiratory burst activities began to return to the level of resident peritoneal cell function. These data suggest two possibilities: (a) direct neutrophil activity alone was responsible for the increased peritoneal cell function observed; and/or (b) indirect neutrophil interaction with macrophages was necessary for these elevated functions.

In summary, these studies indicate that i.p. administration of tolmetin, in some instances, elevated leukocyte functions involved in the clearance of infection. For example, post-surgical administration of tolmetin extended the length of time after surgery that the phagocytic capability of peritoneal leukocytes was enhanced. In addition, tolmetin administration further elevated the surgically-induced increase in superoxide anion release by peritoneal exudate cells and macrophage tumoricidal activity. Therefore, perioperative administration of tolmetin should not increase the risk of infection to the host. These alterations in peritoneal leukocyte function may be the result of a reduction in prostaglandin synthesis by tolmetin.

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# Tumor Growth and Dissemination After Laparotomy and CO<sub>2</sub> Pneumoperitoneum: A Rat Ovarian Cancer Model

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**Objective:** To compare tumor growth, intraperitoneal implantation, and abdominal wall metastasis after laparotomy and CO<sub>2</sub> pneumoperitoneum in a rat ovarian cancer model.

**Methods:** To mimic intraoperative rupture of an ovarian tumor in a syngenic rat ovarian carcinoma model, 10<sup>5</sup> malignant cells were injected intraperitoneally after a 5-cm vertical midline laparotomy or after the insufflation of a CO<sub>2</sub> pneumoperitoneum achieved with 4 mmHg or 10 mmHg intra-abdominal pressure. Two weeks later, the intraperitoneal tumor growth and the tumor dissemination were evaluated semiquantitatively with a scoring system. The scores attributed to each organ were added to calculate the dissemination score of each animal.

**Results:** The mean ( $\pm$  SD) dissemination score was 83.4  $\pm$  12 in the laparotomy group and 67.3  $\pm$  16 and 71.9  $\pm$  17 in the 4 and 10 mmHg CO<sub>2</sub> pneumoperitoneum groups, respectively ( $P < .01$ ). The scores for the peritoneum were 21.8  $\pm$  3.8 in the 10 mmHg pneumoperitoneum group and 18  $\pm$  2.4 in the laparotomy group ( $P < .01$ ). In the laparotomy group, the implant found along the midline scar accounted for a mean of 62.6  $\pm$  15% of the peritoneal score, whereas the trocar site metastases did not influence the peritoneal score in the pneumoperitoneum groups. The incidence of wound metastasis was 96% in the laparotomy group and 55% and 54% in the 4 mmHg and 10 mmHg pneumoperitoneum groups, respectively.

**Conclusion:** In this model, tumor growth was greater after laparotomy than after laparoscopy, but peritoneal tumor dissemination was more severe after CO<sub>2</sub> pneumoperitoneum. (Obstet Gynecol 1998;92:104-8. © 1998 by The American College of Obstetricians and Gynecologists.)

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*A BD IX rat ovarian adenocarcinoma cell line was kindly provided by the Tumorbank Deutsches Krebsforschungszentrum (Pr Friedrich, Dr Peschke, Heidelberg, Germany).*

Laparoscopic management of ovarian cancer is highly controversial. Recent surveys have confirmed that inadequate laparoscopic management of adnexal masses might worsen the prognosis of an early ovarian cancer.<sup>1,2</sup> Volz et al<sup>3</sup> suggested that in a nude mice model, after CO<sub>2</sub> laparoscopy, tumor dissemination and tumor growth were worse than after laparotomy. Several animal studies designed by general surgeons, performed with colon cancer or mammary adenocarcinoma cell lines, showed a higher risk of trocar site metastasis after laparoscopy, but greater tumor growth after laparotomy.<sup>4-11</sup>

We performed a MEDLINE search encompassing the years 1992-1997 using the keywords "cancer" and "laparoscopy," "cancer" and "peritoneum," and "ovary" and "laparoscopy" and found no study performed with an ovarian cancer cell line in a syngenic animal model. Therefore, we designed the present study to compare intraperitoneal tumor growth after laparotomy and CO<sub>2</sub> pneumoperitoneum in an ovarian cancer model.

## Materials and Methods

One hundred twenty-two 12-week-old inbred immunocompetent BD IX rats (Iffa Credo, l'Arbresle, France), weighing between 190 and 220 g, were acclimatized for 7 days to controlled laboratory conditions (temperature 22-24°C, humidity 50 to 60%, 12 hours light, 12 hours dark) before the study. Animals were fed a standard laboratory diet ad libitum. The protocol was approved by the committee on animal research of the Clermont Ferrand I University (Clermont Ferrand, France).

BD IX rat ovarian adenocarcinoma cells, stored in liquid nitrogen until used, were cultured in RPMI 1640

**Table 1.** Scoring System for the Dissemination Score

Number of nodules	Diameter		
	≤2 mm	2-5 mm	≥5 mm
1-4	1	2	3
5-10	4	5	6
11-20	7	8	9
>20	10	11	12

(Kibbutz Beit Haemek, Israel) supplemented with 10% fetal calf serum (Bayer Diagnostics, Puteaux, France), 1% penicillin 5000 IU/mL, 1% streptomycin 5000 IU/mL, and 1% L-glutamine 200 mmol/L. After 48 hours, a monolayer confluent culture was obtained. Before injection in rats, the tumor cells were trypsinized at 37°C for 5 minutes and then centrifuged for 10 minutes at 800 g. The cells were resuspended in the culture medium, counted, and cell viability was confirmed by Trypan blue exclusion test. The cells were diluted to a concentration of  $10^5$ /mL, stored at room temperature, and injected within 3 hours of being obtained.

Thirty animals were used in preliminary experiments. Using 10 rats and a 1.5-mm diameter laparoscope (Karl Storz, Tuttlingen, Germany), we found that below 4 mmHg, it was difficult to achieve a satisfactory inspection of the peritoneal cavity and that a pressure greater than 10 mmHg caused breathing difficulties. Using 20 other animals, we found that the injection of  $10^6$  cells in 1 mL induced in 1 week a peritoneal carcinomatosis that could not be quantified, whereas  $10^5$  cells per mL induced a severe peritoneal carcinomatosis in 2 weeks that could be quantified using a semiquantitative scoring system (Table 1). Preliminary experiments showed that the diameter of the peritoneal metastasis ranged from 2 mm to greater than 10 mm. We concluded that to study tumor growth and peritoneal dissemination, all the peritoneal metastasis should be included in the score. Therefore, we changed the method used by Bouvy et al,<sup>7</sup> scoring each organ between 0 and 5 according to estimated tumor diameter, to adding the scores of each group of nodules. The dissemination score of each animal was calculated by adding the scores of all the organs evaluated. In these preliminary experiments, pathologic examination was used routinely to confirm the macroscopic diagnosis of tumor metastasis. During the study, pathologic examination was used in very small or atypical implants.

On the basis of the mean animal score ( $46.2 \pm 14.06$ ) obtained in the preliminary  $10^5$  group, the sample size was calculated to have a power of .90 with an  $\alpha = .05$ , to detect an increase of 25% in the animal dissemination score. All rats were anesthetized using 50 mg of Zoletil

20 (Laboratoire Reading, Wissous, France) per kilogram intramuscularly. The animals were shaved, placed in the supine position, and operated on under sterile conditions. On the day of surgery, the animal coordinator, who was not involved in surgery, gave an identification number to each animal. Animals were operated on in groups of four. Four cards corresponding to the treatment groups were placed in a box and mixed by the animal care coordinator, who picked the cards to assign each animal a treatment group randomly. In this way the procedures were performed in a random order for each group of four animals.

Ninety-two animals were included in the study, 20 in the control group and 24 in each of the other groups. In the controls, the cells were injected using a 1.5-mm diameter Veress needle (Karl Storz, Tuttlingen, Germany). The intraperitoneal location of the needle was checked by the syringe safety test used in our department.<sup>12</sup> In the laparotomy group, a 5-cm vertical midline laparotomy was performed. Manual manipulations were used to exteriorize the small bowel and the caecum. After 5 minutes, the bowel was returned and the tumor cells were injected in the right lower quadrant. Forty minutes later, the abdomen was closed in two layers with continuous 3-0 Vicryl sutures (Ethicon Inc, Sommerville, NJ).

In the pneumoperitoneum groups, the CO<sub>2</sub> pneumoperitoneum was created using a Verres needle placed in the midline as previously described here. An electronic insufflator (Electronic laparoflator 26430020, Karl Storz, Tuttlingen, Germany) was used to control the insufflation pressure, which was 4 mmHg and 10 mmHg in the low- and high-pressure groups, respectively. On the insufflator, the maximal CO<sub>2</sub> flow was set at 1 L/min. The CO<sub>2</sub> was not warmed. To simulate the CO<sub>2</sub> leakage observed in clinical situations and to avoid incidental increases in intraabdominal pressure, a 20-gauge needle was inserted in the midline between the umbilicus and the pubis. After 5 minutes of pneumoperitoneum, the 20-gauge needle was used to inject the tumor cells in the right lower quadrant of the abdomen. After 45 minutes, the pneumoperitoneum was exsufflated and the skin incisions closed with 3-0 Vicryl.

After 2 weeks, the animals were killed by cervical dislocation under general anesthesia. The skin was removed. Trocar sites and laparotomy incision were evaluated macroscopically. Abdominal wall metastasis was scored as present or absent. When present, its largest dimension was used for statistical analysis. The peritoneal cavity was evaluated through a xyphopubic vertical midline incision. Ascites was aspirated and measured with a 5-mL syringe.

Fisher exact test was used for data presented in

**Table 2.** Incidence and Maximum Dimension of Wound Metastasis

Group	n	Metastasis	Largest dimension (mm)*
		n (%)	
Control	18	16 (89) <sup>†</sup>	3.6 ± 2.7
4 mmHg pneumoperitoneum	22	12 (55)	2.4 ± 3.2
10 mmHg pneumoperitoneum	24	13 (54)	2.5 ± 3.4
Laparotomy	23	22 (96) <sup>‡</sup>	25.2 ± 10.1 <sup>§</sup>

\* Data are presented as mean ± SD.

<sup>†</sup>  $P = .04$ .

<sup>‡</sup>  $P = .002$  when compared with each of the pneumoperitoneum groups using the Fisher exact test.

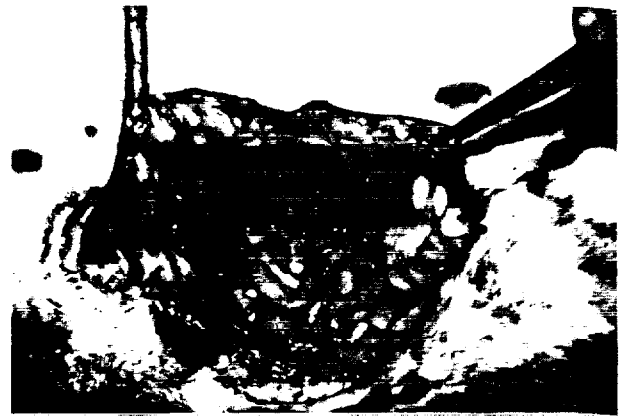
<sup>§</sup>  $P < .001$  when compared with each of the other groups using Student *t*-test.

contingency tables and Student *t*-test for continuous variable. The Kruskal-Wallis *H* test and Mann-Whitney *U* test were used when the distribution was not normal. Differences were considered statistically significant if  $P < 0.05$ .

## Results

Five rats died within the first 24 postoperative hours, two in the control group, two in the low pressure pneumoperitoneum group, and one in the laparotomy group. Autopsies indicated no surgical complications. These animals were excluded from the study. After 2 weeks, a diffuse tumor growth was observed in all rats. The volume of ascites was always less than 4 mL.

The incidence and the largest diameter of wound metastases are presented in Table 2. The animal dissemination scores and the organ scores are listed in Table 3. When compared with each of the other groups, the animal dissemination score in the laparotomy animals was significantly higher than in the 4 mmHg or 10 mmHg pneumoperitoneum groups and the controls.

**Figure 1.** Dissemination after laparotomy. Note the large peritoneal nodule along the midline scar.

The animal dissemination scores in each of the pneumoperitoneum groups were significantly higher than the controls. There was no statistical difference between the groups for the omentum, the scrotal fat, the spleen, and the kidneys (data not shown). Compared with each of the other groups, the scores for peritoneum and bowel were significantly lower in the controls. The score for peritoneum was significantly higher in the high pressure pneumoperitoneum group than in the laparotomy and low-pressure groups. In the laparotomy group, the peritoneal implant found along the midline scar accounted for a mean of  $62.6 \pm 15\%$  of the peritoneal score, whereas in the pneumoperitoneum groups, the trocar site metastases were less than 5 mm in diameter and did not influence the peritoneal score. Examples of peritoneal dissemination after laparotomy and pneumoperitoneum are shown in Figures 1 and 2, respectively.

**Table 3.** Organ and Dissemination Scores\* Observed After Intraperitoneal Injection of  $10^5$  Rat Ovarian Cancer Cells

	Control n = 18	4 mmHg Pneumoperitoneum n = 22	10 mmHg Pneumoperitoneum n = 24	Laparotomy n = 23	P <sup>†</sup>
Peritoneum	7.4 ± 4.1 (2-15)	18.8 ± 5.2 (6-27) <sup>‡</sup>	21.8 ± 3.8 (15-30) <sup>‡*</sup>	18 ± 2.4 (13-21) <sup>§</sup>	0.001
Right diaphragm	5.9 ± 3.4 (2-12)	6.9 ± 3.9 (1-15)	7.6 ± 2.9 (2-12) <sup>§</sup>	10.0 ± 3.0 (5-17) <sup>§</sup>	0.001
Left diaphragm	5.1 ± 3.1 (1-12)	7.0 ± 3.7 (2-15)	6.1 ± 4.3 (1-21) <sup>§</sup>	9.0 ± 3.0 (5-15) <sup>§</sup>	0.002
Liver	0.6 ± 0.9 (0-3)	1.1 ± 1.1 (0-4)	1.6 ± 1.5 (0-5)	3.0 ± 2.2 (0-9)	0.001
Stomach	0.2 ± 0.4 (0-1)	1.2 ± 1.6 (0-6)	1.6 ± 2.8 (0-10)	1.1 ± 1.7 (0-6)	0.03
Bowel	6.8 ± 4.0 (0-18)	10.8 ± 3.6 (6-18)	11.5 ± 3.5 (4-21) <sup>§</sup>	16.7 ± 5.9 (5-24) <sup>§</sup>	0.001
Animal score <sup>‡</sup>	44.2 ± 13.8 (20-68)	67.3 ± 16 (25-92)	71.9 ± 17 (48-117) <sup>§</sup>	83.4 ± 12 (64-106) <sup>§</sup>	0.001

\* Data are presented as mean ± standard deviation (range).

<sup>†</sup> Comparison of all the groups using a Kruskal-Wallis *H* test.

<sup>‡</sup> Comparison with a Mann-Whitney *U* test.  $P < .05$ .

<sup>§</sup> Comparison with a Mann-Whitney *U* test of 10 mmHg and laparotomy groups,  $P < .01$ .

<sup>¶</sup> Comparison with a Mann-Whitney *U* test of the 10 mmHg and laparotomy groups,  $P < .01$ .

<sup>‡</sup> This score was obtained by adding the score of all the intra-abdominal organs including the omentum, the scrotal fat, the spleen, and the kidney, which are not presented here.



**Figure 2.** Dissemination after a 10 mmHg pneumoperitoneum. Note the numerous peritoneal nodules.

## Discussion

As in other studies performed with different cell lines and tumor models,<sup>5,6,9,10</sup> we found greater tumor growth after laparotomy than after pneumoperitoneum. In contrast, Volz et al<sup>3</sup> reported a greater tumor growth after laparoscopy. These differences might be related to the models used. Volz et al<sup>3</sup> compared a 2-cm midline laparotomy with an 8-mmHg pneumoperitoneum in nude mice. Allendorf et al<sup>5</sup> compared a xyphopubic incision with a 6 mmHg pneumoperitoneum in rats. Our results should be interpreted cautiously. The higher dissemination score observed with laparotomy was partly explained by the higher score of the bowel, which was exteriorized for 5 minutes, and by the large peritoneal metastasis found along the midline incision, which is observed only in cell seeding models<sup>7</sup> and may be facilitated by the anatomic position in quadrupeds. However, the ability to perform extensive surgical procedures without exteriorization of the bowel is an advantage of laparoscopic approach.

The peritoneal tumor implantation pattern was different after laparotomy and pneumoperitoneum. In the laparotomy group, the implants found along the midline scar accounted for  $62.6 \pm 15\%$  of the peritoneal score, whereas in the pneumoperitoneum groups, a widespread tumor dissemination was observed (Figures 1 and 2). As the prognosis of an ovarian cancer is more related to the dissemination of the tumor than to the volume of the mass, this pattern of intraperitoneal tumor implantation could be interpreted as a strong argument against a laparoscopic approach. Similar tumor implantation patterns were reported by others.<sup>3,11</sup>

As the peritoneal score was significantly higher in the 10 mmHg group than in the 4 mmHg group (Table 3), the insufflation pressure appeared as a possible cause of peritoneal dissemination. Bouvy et al<sup>7</sup> reported a

smaller tumor growth after gasless laparoscopy than after CO<sub>2</sub> laparoscopy with a 6-mmHg pressure, whereas Watson et al<sup>8</sup> found no difference between a gasless and a CO<sub>2</sub> group with a 2-mmHg pressure. In rats, a 15-mmHg intra-abdominal pressure induced jejunal ischemia.<sup>13</sup> Berguer et al<sup>14</sup> recently reported that in rats, CO<sub>2</sub> pneumoperitoneum greater than 10 mmHg caused significant respiratory acidosis. We observed no breathing difficulties in the 10 mmHg pneumoperitoneum group, but we used a 20-gauge needle to simulate the CO<sub>2</sub> leakage and to avoid incidental increase in intra-abdominal pressure. However, further studies are necessary to confirm that our results are important for surgical practice, which differs in that procedures are longer, pressures used are lower, and their consequences are partly prevented by controlled ventilation.

In the present study, wound metastases were more common and larger with laparotomy. Similar results were reported by Bouvy et al<sup>7</sup> using a cell seeding model. In contrast, most studies performed with solid tumor models showed an increased incidence of trocar site metastasis after CO<sub>2</sub> laparoscopy.<sup>4,6</sup> These differences are probably related to the model used. Our results provide three arguments to suggest that trocar site metastases are more likely caused by direct contamination of the wound than by an aerosol of malignant cells.<sup>15</sup> First, in the small rat peritoneal cavity, malignant cells injected in 1 mL were more likely to induce direct contamination of the laparotomy scar than contamination of the trocar sites when the peritoneal cavity was enlarged by the pneumoperitoneum. Second, the 89% incidence of wound metastasis observed in the control group was probably caused by direct contamination, which occurred when the needle was removed immediately after the injection. Third, the incidence of trocar site metastasis was not increased by a higher intraperitoneal pressure. Two experimental studies showed a decreased incidence of trocar site metastasis after gasless laparoscopy.<sup>7,8</sup> However, this has to be confirmed by clinical data, as the larger port sites used during gasless procedures may increase the risks of direct contamination.

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# Effects of laparoscopy on intraperitoneal tumor growth and distant metastases in an animal model

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**Background and aims.** Laparoscopic surgery for colorectal cancer is currently being evaluated in humans. The aim of this study was to examine the effect of laparoscopy on intraperitoneal tumor growth and distant metastases in an animal model. We also examined the effect of combining laparotomy with laparoscopy and on infusing the peritoneal cavity with normal saline solution (NaCl), water, and sodium hypochlorite after laparoscopy on intraperitoneal tumor growth.

**Material and methods.** Female Fischer rats were given MTLn3 adenocarcinoma cells by intraperitoneal injection to produce intraperitoneal tumor growth and by tail vein injection to produce lung metastases. A pneumoperitoneum was then induced to a pressure of 8 mm Hg with carbon dioxide (CO<sub>2</sub>), helium, or room air. After this, animals were allowed to either recover or underwent laparotomy or infusion of NaCl, water, or sodium hypochlorite before recovery, depending on the experiment. At 21 days all animals were killed and intraperitoneal tumor growth was assessed by counting the number of peritoneal and serosal nodules and by weighing the omental pad of tumor. Lung metastases were assessed by counting the number of metastases after fixation.

**Results.** Laparoscopy caused a marked intraperitoneal dissemination of tumor with a median of 17 (10 to 20) peritoneal and serosal nodules for CO<sub>2</sub>, 19.5 (12.5 to 25) for helium, and 15.0 (9.5 to 17.7) for room air compared with 0 (0 to 1) for controls ( $P < .0001$ ). The weight of omental tumor was also significantly increased ( $P < .02$ ) in the CO<sub>2</sub>, helium, and room air groups. Infusion with NaCl, water, or sodium hypochlorite had no effect on tumor dissemination after laparoscopy. The combination of laparoscopy and laparotomy caused a significant reduction ( $P < .05$ ) in the number of peritoneal nodules but had no significant effect on omental tumor growth. Laparoscopy also had no effect on the number of pulmonary metastases induced compared with controls.

**Conclusions.** This study shows that laparoscopy promotes intraperitoneal dissemination of tumor. This effect is independent of the insufflating gas used and is not affected by use of a cytotoxic agent. The use of gasless laparoscopy should be encouraged by those undertaking curative laparoscopic surgery for colorectal cancer. (Surgery 1999;126:35-40.)

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IN 1991, AS LAPAROSCOPIC CHOLECYSTECTOMY was being accepted as a new technique in many centers, Jacobs et al<sup>1</sup> and Redwine and Sharpe<sup>2</sup> described laparoscopic right and left hemicolectomy, abdominoperineal resection, and stoma formation for both benign and malignant disease. This generated interest in laparoscopic colorectal surgery elsewhere, and reports from both the United Kingdom and the United States<sup>3-6</sup> confirmed the feasibility of these

techniques. Although the ability to perform laparoscopic colorectal resection cannot be questioned, controversy regarding its oncologic safety remains. Enthusiasts claim that the principles of oncologic surgery are strictly adhered to, with resection margins, tumor clearance, and lymph node harvest comparable to those of conventional open surgery.<sup>7</sup> However, as yet no long-term follow-up of patients after laparoscopically assisted surgery for colorectal cancer is available.

Of particular concern has been the high rate of wound recurrence and port-site metastases reported after a short follow-up period.<sup>8</sup> The first reports of port-site metastases were observed after staging procedures for intraperitoneal cancers.<sup>9-13</sup> Such lesions have also been reported after cholecystectomy<sup>14-17</sup> and now there are many reports of port-site metastases after colorectal cancer surgery.<sup>12-14,16-24</sup>

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In a recent review of the literature by Wexner and Cohen<sup>25</sup> the authors estimated that 6.5% of patients undergoing curative colorectal cancer surgery have port-site metastases. Although this is likely to be an overestimate, it is clear that wound recurrence after open surgery remains rare and is thought to be less than 1%.<sup>26</sup>

Little is known about the effect of laparoscopy on intraperitoneal tumor growth. The aim of this study was to examine the effect of laparoscopy with different gases on tumor growth after intraperitoneal and intravenous injection of tumor in an animal model. We also examined the effect of combining laparoscopy and laparotomy on tumor growth in this model. Finally, because normal saline solution (NaCl) and occasionally sterile water are used as lavage fluids during laparoscopic surgery, we examined the effect of infusion of these fluids and the cytotoxic agent sodium hypochlorite on intraperitoneal tumor growth after laparoscopy.

#### MATERIAL AND METHODS

**Animals.** Female Fischer rats aged 8 to 12 weeks were obtained from Harlan UK Ltd. Rats were maintained in the University of Glasgow animal facility under appropriate conditions and were allowed food and water ad libitum. All work was performed under the provisions of the "Animals (Scientific Procedures) Act 1986" and the supervision of the Home Office.

**Tumor cells.** The cell line was the MtLn3 clone of the rat adenocarcinoma cell line originally derived by Neri and Nicholson, M. D. Anderson Hospital and Tumour Institute, Houston, Tex.<sup>27</sup> This is derived from the mammary adenocarcinoma line 13762NF, induced by dietary administration of 7,12-dimethylbenz[a]-anthracene, and is known to have a high metastatic potential. It is syngenic for the F344 Fischer rat. Cells have been kept in liquid nitrogen. Each batch of frozen cells was passaged no more than 6 times to prevent phenotypic drift. The medium used was Hanks F10 and Dulbecco's modified Eagles' medium (Life Technologies, Paisley) with 10% fetal calf serum and L-glutamine. Cells were grown to confluence in 5% carbon dioxide (CO<sub>2</sub>) at 37°C. They were then washed with phosphate-buffered saline solution and trypsinized with 0.2% trypsin-EDTA (ethylenediaminetetraacetic acid) solution (Life Technologies). They were washed 3 times in medium by centrifugation at 1200 revolutions/min and resuspended in fresh medium at the appropriate concentration. Viability was assessed by trypan blue exclusion. All cell suspensions were greater than 90% viable and were used within 2 hours of preparation.

**Study 1: effect of laparoscopy on intraperitoneal tumor spread.** The pattern of intraperitoneal spread of MtLn3 has been previously described.<sup>28</sup> Briefly, after intraperitoneal injection a tumor nodule develops at the injection site. In addition, there are multiple small parietal peritoneal deposits, particularly in the subdiaphragmatic area. The omentum becomes diffusely infiltrated with tumor, but there are few or no visceral deposits and no solid-organ metastases.

Seventy animals were divided into 4 groups. General anesthetic was induced with oxygen-halothane and animals were maintained with 2% halothane by a nose cone. All animals received an intraperitoneal injection of  $1 \times 10^4$  cells of MtLn3 in 1 mL of medium. Group 1 (controls) had intraperitoneal tumor injection and anesthetic only. Group 2 had a CO<sub>2</sub> pneumoperitoneum induced by inserting an 18-gauge needle in the midline subumbilically and insufflating to a pressure of 8 mm Hg (Wolff automatic insufflator). This pressure produces a tense pneumoperitoneum without causing diaphragmatic splinting in the nonintubated animal. Group 3 had a helium pneumoperitoneum induced in the same manner, whereas group 4 had a pneumoperitoneum induced with room air. A pressure of 8 mm Hg was maintained for 15 minutes. After this time the abdomen was allowed to deflate through the 18-gauge needle, which was then removed and the animals recovered. Any remaining low-pressure pneumoperitoneum was reabsorbed over the succeeding 24 hours. All animals were killed at 21 days, or sooner if the clinical condition deteriorated.

**Assessment of intraperitoneal tumor spread.** Tumor spread was assessed by careful counting of peritoneal and serosal nodules and weighing the excised omentum. Specimens were sent for histopathologic study to confirm the presence of tumor.

**Study 2: effect of laparoscopy and laparotomy on intraperitoneal tumor growth.** Because most laparoscopic procedures for colorectal cancer combine laparoscopy with a small laparotomy wound, we examined the effects of both laparoscopy and laparotomy on intraperitoneal tumor growth. Thirty animals were randomized into 3 groups. All were anesthetized as above and received  $1 \times 10^4$  MtLn3 cells in 1 mL of medium. Group 1 received a CO<sub>2</sub> pneumoperitoneum as previously described to a pressure of 8 mm Hg. Group 2 underwent laparotomy only. Group 3 had a pneumoperitoneum with CO<sub>2</sub> for 15 minutes, followed by a laparotomy. At the end of the procedure the wound was closed in layers with plain catgut suture to the

**Table I.** Effect of laparoscopy on intraperitoneal tumor growth

Group	No.	Omental tumor (g)	Peritoneal nodules
Control	20	4.0 (3.2-5.9)	0 (0-1.0)
CO <sub>2</sub>	20	7.5 (5.8-8.8)	17.0 (10.0-20.0)
Helium	20	6.1 (5.0-8.3)	19.5 (12.5-25.7)
Room air	10	7.4 (3.8-8.6)	15.0 (9.5-17.7)
Statistical significance*		P = .018	P = .0001

\*Compared with controls.

peritoneum and polyglactin mesh (Vicryl, Ethicon, Somerville, NJ) to the skin; all animals recovered. All animals were killed at 21 days, or sooner if the clinical condition indicated. The extent of tumor growth was assessed as previously described, and an assessment of wound tumor was made.

**Study 3: effect of laparoscopy followed by peritoneal infusion on intraperitoneal tumor growth.** Forty F344 rats were randomized into 4 groups. All were anesthetized as above and received  $1 \times 10^4$  MtLn3 tumor cells in 1 mL as an intraperitoneal injection. Group 1 (controls) underwent a CO<sub>2</sub> pneumoperitoneum as previously described. Group 2 underwent pneumoperitoneum followed by infusion of 2 mL of warm NaCl into the peritoneal cavity. Group 3 underwent the same procedure with sterile water, whereas group 4 rats were infused with a solution of 0.3% sodium hypochlorite (Milton). Sodium hypochlorite was chosen for this experiment because it had previously been shown to be the most effective cytotoxic solution both in vitro and in vivo against the MtLn3 cell line.<sup>28</sup> Infusions were left within the abdominal cavity. At the end of the procedure all animals were recovered. Animals were killed at 21 days or sooner if clinical conditions indicated, and tumor load was assessed as above.

**Study 4: effect of laparoscopy on lung metastases.** In this model, after a tail vein injection of MtLn3 tumor cells, lung metastases will develop in all animals. Forty-five animals were randomized into 3 groups: control, CO<sub>2</sub> pneumoperitoneum, and helium pneumoperitoneum. All had general anesthesia induced with oxygen-halothane in a Perspex box and were maintained with 2% halothane by a nose cone. All received a tail vein injection of  $1 \times 10^4$  cells in 0.2 mL of medium. Pneumoperitoneum was then induced as in the first experiment. All animals were killed at 21 days, or sooner if the clinical condition indicated, and lung metastases were counted.

**Assessment of lung metastases.** The metastases were counted in the manner of Wexler.<sup>29</sup> Briefly, the excised lungs were washed with water and inflated with India ink through the trachea. They were

then fixed in alcoholic Bouin's solution. Metastases became white and could be counted at 24 hours.

**Statistics.** Data were collated with use of the Statistics Package for Social Sciences (SPSS, Chicago, Ill). All values are expressed as medians with interquartile ranges. Peritoneal nodules, lung metastases, and omental weights were compared with the Mann-Whitney U-Wilcoxon rank-sum test.

## RESULTS

**Study 1: effect of laparoscopy on intraperitoneal tumor spread.** All animals survived to 21 days and all had diffuse intraperitoneal tumor involving the parietal peritoneal surfaces with gross infiltration of the omentum and with blood-stained ascites at autopsy. Both the CO<sub>2</sub> helium and room air laparoscopy groups had significantly greater omental and peritoneal involvement compared with controls (Table I). There was no significant difference between the CO<sub>2</sub> helium and room air groups. Histopathologic study confirmed the presence of adenocarcinoma at all sites tested.

**Study 2: effect of laparoscopy and laparotomy on intraperitoneal tumor growth.** All animals survived to 21 days and had blood-stained ascites with disseminated intraperitoneal tumor as before. In addition, all animals in the laparotomy group had diffuse infiltration of the wound with tumor. There was no significant difference in the amount of omental tumor in any group nor in the degree of tumor infiltration in the wound. However, both the laparotomy alone group and the laparotomy plus laparoscopy group had significantly fewer peritoneal deposits compared with the laparoscopy alone group (Table II).

**Study 3: effect of laparoscopy followed by peritoneal infusion on intraperitoneal tumor growth.** All animals survived to 21 days and had blood-stained ascites and tumor growth within the peritoneum as described. There were no significant differences in the amount of tumor growth between control and infusion groups (Table III).

**Study 4: effect of laparoscopy on lung metastases.** All animals survived to 21 days and at autopsy all groups had evidence of lung metastases with-

**Table II.** Effect of laparoscopy and laparotomy on intraperitoneal tumor growth

Group	No.	Omental tumor (g)	Peritoneal nodules
Laparoscopy	10	7.2 (4.6-8.1)	13.0 (6.5-18.5)
Laparotomy	10	8.1 (5.7-11.8)	3.0 (1.0-4.0)
Combined	10	5.9 (4.6-8.0)	5.0 (2.5-6.5)
Statistical significance*		NS	P = .05

NS, Not significant.

\*Compared with laparoscopy group.

**Table III.** Effect of laparoscopy followed by peritoneal infusion on intraperitoneal tumor growth

Group	No.	Omental tumor (g)	Peritoneal nodules
Laparoscopy	10	4.6 (3.5-6.1)	14.5 (10.5-17.0)
Water	10	5.3 (3.4-6.8)	11.0 (6.7-14)
NaCl	10	6.1 (3.2-6.2)	10.5 (6.0-13.5)
Sodium hypochlorite	10	6.9 (6.6-7.9)	12.0 (10.5-13.2)
Statistical significance*		NS	NS

NS, Not significant.

\*Compared with laparoscopy group.

**Table IV.** Effect of laparoscopy on lung metastases

Group	No.	Lung metastases
Control	15	5 (3-12)
CO <sub>2</sub>	15	9 (7-10)
Helium	15	6 (6-14)
Statistical significance*		NS

NS, Not significant.

\*Compared with control group.

out gross dissemination of disease. There were no significant differences in the number of lung metastases observed among the CO<sub>2</sub>, helium, and control groups (Table IV).

## DISCUSSION

Our results show that laparoscopy causes dissemination of tumor cells and promotes tumor growth within the peritoneal cavity in an animal model. This effect was independent of the gas used and was significantly reduced when laparoscopy was followed by laparotomy. It was not affected by infusion of sterile water, NaCl, or the cytotoxic agent sodium hypochlorite.

Interestingly, laparoscopy could not be demonstrated to have any effect on distant metastases, indicating that local factors may be the predominant mechanism whereby laparoscopy promotes intraperitoneal dissemination of tumor. It is likely that there is aerosolization of tumor cells within the enclosed space of the peritoneal cavity. In the clinical setting, with repeated introduction and withdrawal of instruments allowing gas to leak through ports, cells may be drawn to the port sites. The

pressure of insufflating gas may force these cells into the peritoneum and port-site wound, allowing them to seed and grow. The fact that in this model laparoscopy insufflation followed by laparotomy significantly reduces the amount of peritoneal tumor growth with little effect on omental tumor growth suggests that prolonged pressure is required to allow seeding and growth of peritoneal nodules to take place.

The concept of tumor cell aerosolization is supported by Knolmayer et al<sup>30</sup> and their large animal model, which showed that there is a constant aerosol of epithelial cells within the peritoneum during laparoscopy and that the number of cells escaping increases with increasing intra-abdominal pressure. Champault et al<sup>31</sup> have shown the presence of clumps of cells in gas exhausted from laparoscopy, the so-called "chimney effect," although no malignant cells have yet been identified in their studies.

Several other workers have investigated the effect of a pneumoperitoneum on intraperitoneal tumor growth with use of cell suspension and solid tumor in small animal models. Jacobi et al<sup>32</sup> showed that the simple act of creating a CO<sub>2</sub> pneumoperitoneum caused increased tumor growth within the peritoneal cavity. Work by Bouvy et al<sup>33,34</sup> using both cell suspension and solid tumor models achieved similar results. In contrast, the results of Hubens and Eyskens<sup>35</sup> showed no difference in the rate intraperitoneal tumor growth between anesthetic controls and CO<sub>2</sub> insufflation. They scored peritoneal involvement with use of a scale described by Eggermont et al<sup>36</sup> that assessed

intraperitoneal involvement on the basis of visual inspection and does not differentiate between tumor growth within the omentum and involvement of the parietal peritoneum. It is interesting to note that, in common with our results, they did not find any significant differences in tumor growth between laparoscopy and laparotomy.

Bouvy et al<sup>33</sup> also investigated the effects of gasless laparoscopy on intraperitoneal tumor growth with use of a solid tumor model and showed that both laparotomy and CO<sub>2</sub> pneumoperitoneum produced significantly greater intraperitoneal tumor growth than gasless laparoscopy did. Port-site metastases were also significantly reduced in the gasless group compared with the CO<sub>2</sub> pneumoperitoneum group. Similar results were achieved by the same authors with a cell suspension of CC531.<sup>34</sup> These results are supported by Watson et al,<sup>37</sup> who demonstrated that the incidence of port-site metastases in a rat model was reduced after gasless laparoscopy compared with insufflation with CO<sub>2</sub>.

In comparing the effects of laparotomy, laparoscopy, and a combined laparoscopy-laparotomy procedure, our results showed that laparoscopy alone produced significantly more parietal peritoneal tumor deposits than did either laparotomy or the combined procedure. There was no significant difference in omental tumor weight among the 3 groups nor in the amount of wound involvement between the laparotomy and combined groups. It is well known that laparotomy has a permissive effect on tumor growth.<sup>38</sup> However, studies generally demonstrate that this effect is less with laparoscopy.<sup>32-35,39</sup> These studies have examined the effects of laparoscopy on tumor growth at a site remote from the peritoneal cavity, such as the animal's flank, and may help to explain some of the differences between their findings and our study.

It might be expected that infusion of the abdominal cavity with sterile water or sodium hypochlorite would result in a reduction of intraperitoneal tumor growth. We have previously shown that sodium hypochlorite is cytotoxic in vitro and in vivo against the MtLn3 cell line.<sup>28</sup> Further work is therefore required to identify a cytotoxic agent that will prevent the growth of tumor that occurs with laparoscopic insufflation. In the meantime, avoidance of insufflation by the use of gasless laparoscopy should be practiced by surgeons undertaking laparoscopic resection for colorectal cancer.

In conclusion, we have shown that laparoscopy promotes the dissemination of tumor growth within the peritoneal cavity and that this effect is independent of the gas used. Use of a cytotoxic agent or

lavage with water or saline solution has no effect on tumor cell dissemination by laparoscopy. Further work is required to investigate the role of instrument and cannula contamination on port-site metastases and to examine what effect laparoscopy may have on peritoneal and serosal surfaces that promote tumor growth.

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